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Title of Thesis: "Pregnancy Specific Glycoprotein 23 binds to CD151 and induces the secretion of IL-10 and TGF-β_1 in murine macrophages"

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Master of Science
2007

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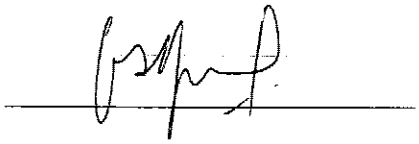

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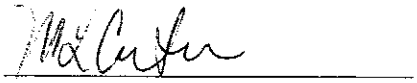

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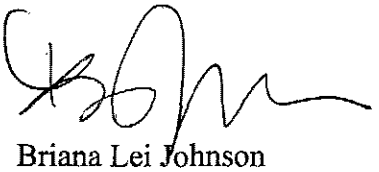

Briana Lei Johnson
Graduate Degree Program in Pathology
Uniformed Services University of the Health Sciences

Abstract

Title of Thesis:	Pregnancy Specific Glycoprotein 23 binds to CD151 and induces the secretion of IL-10 and TGF-β_1 in murine macrophages
Name, degree, year:	Briana Lei Johnson Master of Science 2007
Thesis directed by:	Gabriela S. Dveksler, Ph.D. Professor Department of Pathology Uniformed Services University of the Health Sciences

Pregnancy-specific glycoproteins are placentally secreted proteins present in the maternal circulation. They are the most abundant fetal protein in late pregnancy and correlate with human pregnancy success.

Previously, we identified the tetraspanin CD9 as the receptor for PSG17. This interaction is required for PSG17 mediated anti-inflammatory cytokine production. The present research analyzes the functions and receptor binding of PSG23 in relation to PSG17.

Treatment of RAW 264.7 cells or C57BL/6 macrophages, wild-type or CD9-deficient, with PSG23 induced IL-10 and TGF-β_1, suggesting that PSG23 does not require CD9 for its function. Pull-down, panning, and ELISA revealed that CD9 is in fact not PSG23's receptor. Other tetraspanins were examined in attempt to discover the receptor for PSG23; FACS and ELISA indicate preferential binding to CD151.

While functionally similar to PSG17, PSG23 induced cytokine production isn't mediated through CD9, but possibly CD151, indicating a potential role for tetraspanins in PSG-mediated pregnancy success.

PREGNANCY SPECIFIC GLYCOPROTEIN 23 BINDS TO CD151 AND INDUCES THE SECRETION OF IL-10 AND TGF-β_1 IN MURINE MACROPHAGES

by

Briana Lei Johnson

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For my Mother,
You are my inspiration

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PART TWO. RESEARCH

Glossary

AA: arachidonic acid

APC: antigen presenting cells

BMPs: bone morphogenetic proteins

CEA: carcinoembryonic antigen

CEACAM: CEA-related cell adhesion molecule

COX-2: cyclooxygenase 2

CPE: core promoter element

CTB: cytotrophoblast

dNK: decidual natural killer cell

EC1: extracellular loop 1

EC2: extracellular loop 2

GDFs: growth and differentiation factors

hCG: human chorionic gonadotropin

HHT: 12-hydroxyheptadecatrienoic acid

HLA: human leukocyte antigen

hPL: human placental lactogen

IDO: indoleamine 2,3-dioxygenase

IgC: immunoglobulin constant

IgSF: immunoglobulin superfamily

IgV: immunoglobulin variable

IL: interleukin

iNOS: inducible nitric oxide synthase

KLF: Krüppel-like factor

LPS: lipopolysaccharide

mAb: monoclonal antibody

MHC: major histocompatibility complex

MW: molecular weights

OVA: ovalbumin

PG: prostaglandin

PGI_2: prostacyclin

PSG: pregnancy specific glycoprotein

RGD: arginine-glycine-aspartic acid

RSA: recurrent spontaneous abortions

SP1: specificity protein 1

STB: syncytiotrophoblast

TGF-β: transforming growth factor-beta

TNF-α: tumor necrosis factor-alpha

TXA: thromboxane A_2

uNK: uterine natural killer cell

PART ONE

INTRODUCTION

1 Significance: Statistical analysis of Pregnancy outcomes

Infertility is a global concern; there are approximately 6 million pregnancies per year with only 4 million resulting in live births in the United States alone [1]. In fact, one in two hundred couples experience 2 or more consecutive miscarriages, 50 percent of which are believed to result from immune dysfunction [2].

Maternal fetal rejection accounts for a considerable proportion of miscarriages [3]. Successful human pregnancy requires maternal immune tolerance of the semi-allogeneic conceptus for the duration of pregnancy, defying the precepts of immunology [4]. During this phenomenon called pregnancy, the maternal immune response is uncompromised and fully functional making it quite remarkable for the fetus to evade rejection. Both fetal and maternal organs and tissues contribute to fetal tolerance through the reprogramming of maternal physiology [5]. One type of fetal protein abundantly secreted into the maternal circulation is pregnancy specific glycoprotein (PSG), which is believed to aid and abet in the regulation of the maternal immune response during pregnancy [6].

2 PSGs are a member of the CEA Family

The human carcinoembryonic antigen (CEA) family was fully characterized in 1987 and to date comprises 29 different genes clustered on chromosome 19q13.2 [7]. (Figure 1) The CEA family belongs to the Immunoglobulin superfamily (IgSF) and based on nucleotide sequence comparisons consists of three subgroups, namely CEA-related cell adhesion molecule (CEACAM), pregnancy specific glycoprotein, and a third subgroup [7-9]. The time of divergence for CEACAM and PSG subgroups was likely

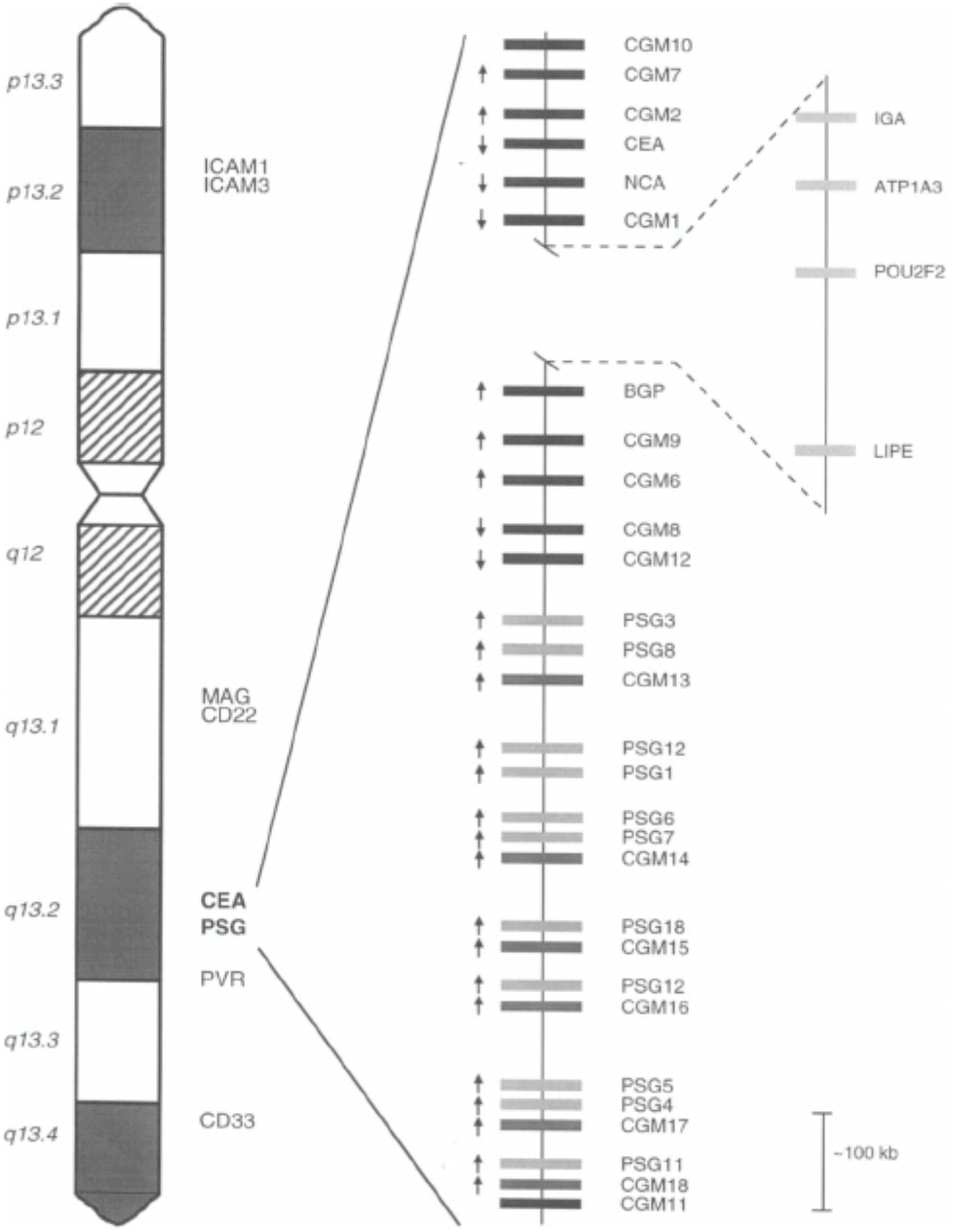


Figure 1. Human CEA gene family clustered on chromosome 19q13.2.

All 29 members of the CEA subgroups are shown to the right of the chromosome. The 11 PSG genes are organized in tandem. Genes encoding the third subgroup (CGM13-18) are interspersed among the PSG genes [7].

to be 107.7 ± 17.1 million years ago [10]. The CEACAM subgroup contains 12 members, 4 of which are pseudogenes and the third subgroup is comprised solely of pseudogenes (six), while all eleven PSG members are expressed. Although all PSG genes may be transcriptionally active, allelic variants with stop codons in the N-domain exon exist for PSG7, 8, and 12 [7].

CEA-related proteins are highly glycosylated (30-60% carbohydrate) [11] and can be either membrane-bound or actively secreted [7, 12]. In contrast to CEACAMs, which are primarily membrane bound, PSGs are secreted because they lack hydrophobic C-terminal domains necessary for membrane anchorage [13]. The CEA subgroups contain a leader peptide which is cleaved from the mature protein [7], one N-terminal immunoglobulin variable (IgV)-like domain, and between zero and six immunoglobulin constant (IgC)-like domains, all encoded by separate exons [7, 12, 14]. The IgC-like domains may either be type A or B containing 93 and 85 amino acids, respectively [7].

While the *in vivo* functions for all CEA family members are still unknown [12], biological activity seems to be mediated through receptor/ligand binding within the N-domain of these proteins [6, 7, 15-17]. As a family, the CEA subgroups have been identified in various cell types (epithelial, endothelial, granulocytes, macrophages, B cells, activated T cells, and trophoblasts) [18] and implicated in a vast array of biological processes, ranging from tumorigenesis to maternal immune modulation [12]. The cellular expression of the CEACAM subgroup is coincident with its biological roles; they are involved in cellular adhesion, colorectal tumor progression, innate immunity, and signal transduction [7, 19, 20]. The trophoblast-specific PSGs have been shown to induce anti-

inflammatory cytokines during pregnancy [15-17], possibly reprogramming maternal physiology in support of fetal tolerance [7], thereby reducing preterm labor [21, 22].

3 Human PSGs: Expression, Structure, and Function

3.1 PSG synthesis and expression

Pregnancy specific glycoproteins are a group of highly similar proteins originally isolated from maternal sera as early as 16 days post-conception. Later, it was determined that all human PSGs are secreted at different levels by the placenta [23, 24] into the maternal circulation during pregnancy [25]. Additionally, PSGs have been detected, albeit at low levels, in non-pregnant human serum [26], fetal liver, salivary gland, testis, myeloid cells [25, 27, 28], intestine, and uterus [25]. Immunohistochemical analysis of RNA isolated from bone marrow and peripheral blood cells revealed trace amounts of PSG transcripts in polymorphonuclear cells, monocytes, B lymphocytes, and T lymphocytes that displayed the highest expression level [24].

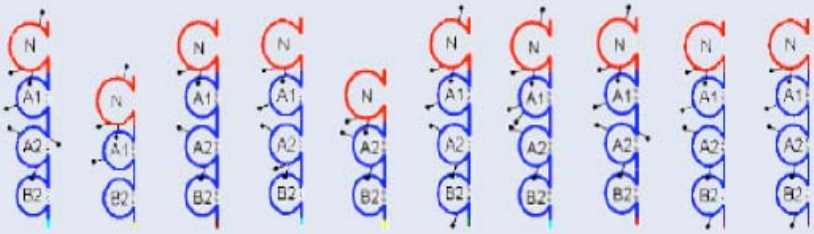
Further characterization of PSG placental expression was attained following the development of a BAP-3 monoclonal antibody (mAb) against the B2 domain of human pregnancy specific glycoproteins. Using the BAP-3 mAb, Zhou et al. established that PSG expression is limited to the syncytiotrophoblast (STB) cells of the placenta, with maximal staining during the first trimester compared to term. Within the STBs, PSG expression was noted in all three intracellular compartments involved in the biosynthetic-secretory pathway, specifically the rough endoplasmic reticulum, the Golgi complex, and secretory vesicles [28]. Furthermore, PSG expression is significantly upregulated during trophoblast differentiation from cytotrophoblast (CTB) to STB [29].

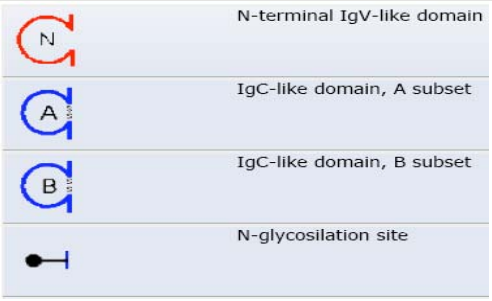
3.2 PSG structure

Originally, PSG was thought to be a single protein and consequently through molecular cloning it was revealed that PSG is in fact encoded by 11 separate genes, namely PSG1-11 [25, 28]. The members of the PSG family are highly similar, displaying 90% homology at the nucleotide level and 85% homology at the amino acid level [25]. All PSGs share similar structural organization [27], containing a 34 amino acid leader peptide (L) that is cleaved upon placental secretion, followed by a 108-109 amino acid IgV-like N-terminal domain, two to three A and B IgC-like domains, containing 93 and 85 amino acids respectively, and a short, 2-13 amino acid, hydrophilic C-terminal tail [7, 27, 28, 30]. This protein organization is coincident to the gene exon organization. The first exon encodes most of the L domain and the second exon encodes the remainder of the L domain along with the N domain, while the A and B domains are encoded discretely by six separate exons [31]. Due to alternate exon splicing, PSGs exist in various domain arrangements, including L-N-A1-A2-B2-C (type I), L-N-A1-B2-C (type IIa), L-N-A2-B2-C (type IIb), L-N-B2-C (type III), and L-A1-B2-C (type IV), resulting in the expression of approximately 30 PSGs during pregnancy [32]. (Figure 2)

Pregnancy specific glycoproteins are highly glycosylated with carbohydrates accounting for approximately 30% of their mass [25]. PSGs isolated from human placenta revealed a series of molecular weights (MW) including, 72, 64, 54, 50, 48, and 36 kDa, by silver staining and/or immunoblotting with anti-PS-β-G serum. The latter 3 represent the unglycosylated forms [26], whereas the higher MW observed results from glycosylation at 4 to 8 prospective N-linked glycosylation sites, with the majority containing between 6 and 7 sites [25].

A

protein	PSG1	PSG2	PSG3	PSG4	PSG5	PSG6	PSG7	PSG8	PSG9	PSG11
domain organisation										
gene	*PSG1*	*PSG2*	*PSG3*	*PSG4*	*PSG5*	*PSG6*	*PSG7*	*PSG8*	*PSG9*	*PSG11*
splice variants	5	1	1	4	1	2	1	1	3	1



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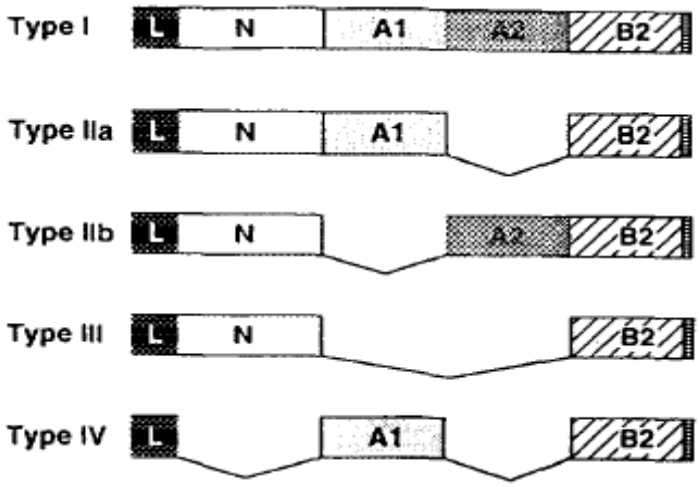

Figure 2. Human PSG domain organization

(a) Human PSGs contain one IgV-like N-terminal domain, followed by 2 or 3 IgC-like domains [33].
(b) Due to alternate splicing, PSG transcripts exist in various domain arrangements [27].

As previously demonstrated by our laboratory, the N domain of both human and murine PSGs is sufficient to elicit biological activity and receptor binding [6, 15-17]. The N domain is composed of 10 beta-sheets, with the greatest level of conservation in the ABED face. The more variable CFG face is believed to be involved in binding, as demonstrated in other CEA family members [34]. Most human PSGs, with the exception of PSG1, 4, and 8 [35], contain a RGD (arginine-glycine-aspartic acid) motif on the solvent exposed loop [30, 34-38] within the CFG face [34]. (Figure 3) The RGD motif forms the minimal functional binding region of integrins and is the cell-binding domain of several extracellular matrix proteins [30, 38], suggesting that this region may be critical for PSG receptor binding and biological activity.

3.3 PSG regulation

Currently, the molecular mechanisms responsible for regulating PSG gene expression have not been fully elucidated [39]. Bocco et al. previously reported that PSG biosynthesis is regulated at the transcriptional level during placental development. The upstream promoter regions of all human PSGs were characterized and display 90% homology. All PSG promoters lack the classical TATA-box, pyrimidine-rich initiator element, and large GC regions [39, 40].

The PSG5 promoter contains a GC-box that acts as a core promoter element (CPE) and interacts *in vitro* with Krüppel-like factor 6 (KLF6). KLF6 is a zinc finger transcription factor highly expressed in human and murine placenta. Additionally, the PSG5 promoter contains a potential binding site for another KLF family member, KLF4 [39, 40], and a site for specificity protein 1 (SP1), a ubiquitous transcription factor [10].

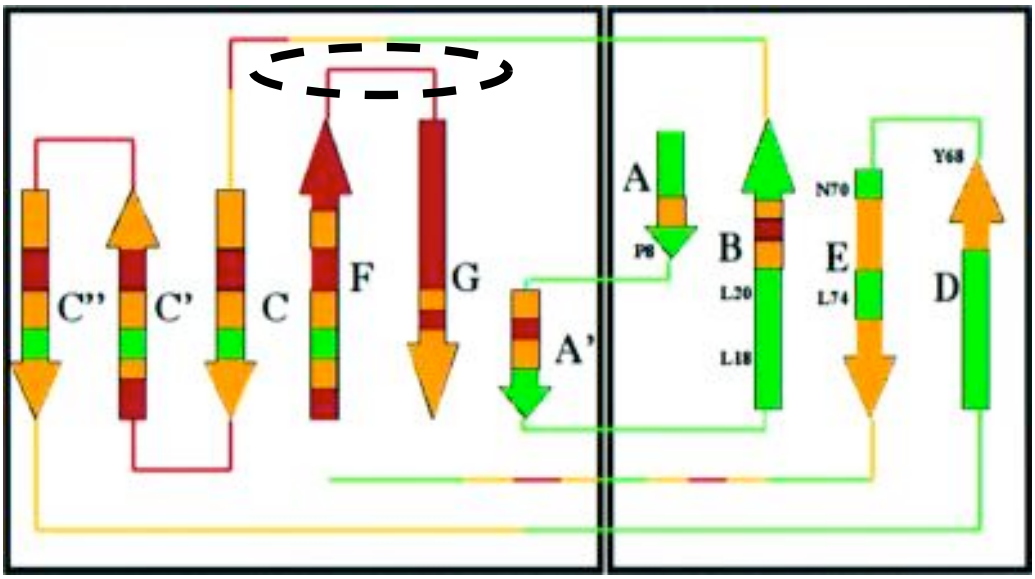

Figure 3. PSG N-terminal domain

The N domains of all members of the CEA family, including PSGs, have 10 beta-sheets, as seen here, with the greatest level of conservation in the ABED face. The more variable CFG face is believed to be involved in binding, as demonstrated in other CEA family members. Most human PSGs have a RGD tripeptide that is aligned on the solvent exposed loop (dashed line) within the CFG face (green – most conserved, red – least conserved, yellow – residues in between) [34].

Interestingly, Sp1 and KLF4 are co-expressed with PSG genes in the STB [39, 40] and upon binding to FP1 and CPE, respectively, they synergistically regulate the transcription of PSG5 [39]. (Figure 4)

3.4 PSG function and relation to pregnancy outcome

PSGs are the most abundant fetal protein present in late pregnancy with concentrations reaching 200-400μg/ml [27]. They are essential for pregnancy success, as low levels correlate with various pathological pregnancy outcomes, including spontaneous abortion, intrauterine growth restriction, pre-eclampsia [41, 42], and fetal hypoxia [43]. Unusually elevated PSG levels have been implicated in pathological conditions as well, i.e. molar pregnancies [44, 45]. The exact function of PSGs remain elusive, however they seem to be essential for the maintenance of normal pregnancy [39].

Their importance is apparent upon administration of anti-human PSG antibodies to cynomolgus monkeys resultant in reduced fertility and increased spontaneous abortion [46]. Also, the expression of PSG11 was significantly lower in the uterine endometrium of women experiencing recurrent spontaneous abortions (RSA) [41]. Another noteworthy finding is the improvement of rheumatoid arthritis with elevated PSG levels during pregnancy [30, 47].

Studies using recombinant (rec) human PSGs demonstrate their ability to induce an anti-inflammatory response that perhaps coincides with the Th2 environment observed during normal pregnancy. Treatment of human monocytes and murine macrophages with PSG1, 6, and 11 induces IL-10, IL-6, and TGF-β_1, but not the pro-inflammatory cytokines IL-1β, TNF-α, or IL-12 [16]. Additionally, recombinant PSG1a is able to

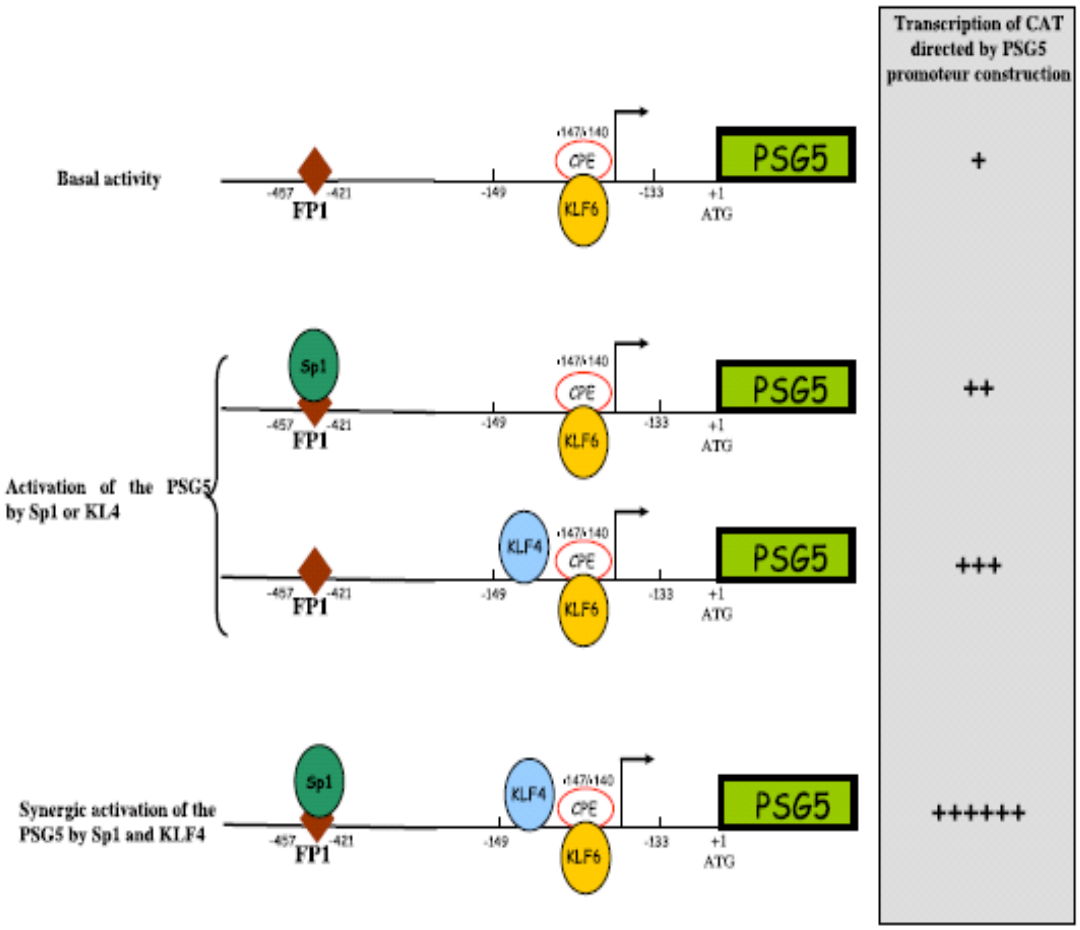

Figure 4. Synergistic activation of PSG5 by KLF4 and SP1

The PSG5 promoter is represented by two binding sites: FP1 (– 457 to – 421) and the region including the CPE (–147 to –140). Three levels of PSG-5 activation are possible: basal activity when KLF6 is bound to the CPE, intermediate induction when KLF4 or Sp1 are bound to CPE or FP1, respectively, and synergic induction when KLF4 and Sp1 are both bound [39].

induce the alternative activation of monocytes [48] and inhibit T cell proliferation [30, 48]. Treatment of BALB/c mice with a vaccinia virus-based vector containing human PSG1a prior to ovalbumin (OVA) immunization results in a Th2 response characterized by high IL-4, IL-5, and IL-10 production [49]. Furthermore, Motran et al. demonstrated that rec-PSG1a successfully upregulated arginase activity, thereby inhibiting inducible nitric oxide synthase (iNOS) activity, in LPS-activated monocytes [48].

4 Murine PSGs: Structure, Expression, and Function

4.1 Murine PSG structure and expression

PSG homologs have been identified in other species with haemochorial placentation as well, including primates, mice, and rats. The murine PSG genes (PSG16-32) [36] are located on the proximal region of the mouse chromosome 7, syntenic to the region of human chromosome 19 containing the human CEA gene family [13, 43, 50]. Murine PSGs are highly glycosylated, as in humans, containing from 6-17 potential N-linked glycosylation sites [36]. Conversely, their domain arrangement is slightly divergent from the human organization. Murine PSGs consist of a varying number of IgV-like domains and only one IgC-like-A domain. The murine N1-terminal domain and carboxyl-terminal-A domain correspond respectively to the human N-terminal and B2 domains [36, 51]. The RGD motif is not present in murine PSGs; however RGD-like motifs (RGE, HGE, and HAE) are aligned in a similar fashion on the solvent exposed loop of the N1 domain [36, 37, 51]. (Figure 5) The amino acid level of conservation is slightly reduced compared to their human counterparts, displaying as low as 59% homology. The A domain displays significantly higher homology (70-88%) than the N1 domain (59-72%) [51].

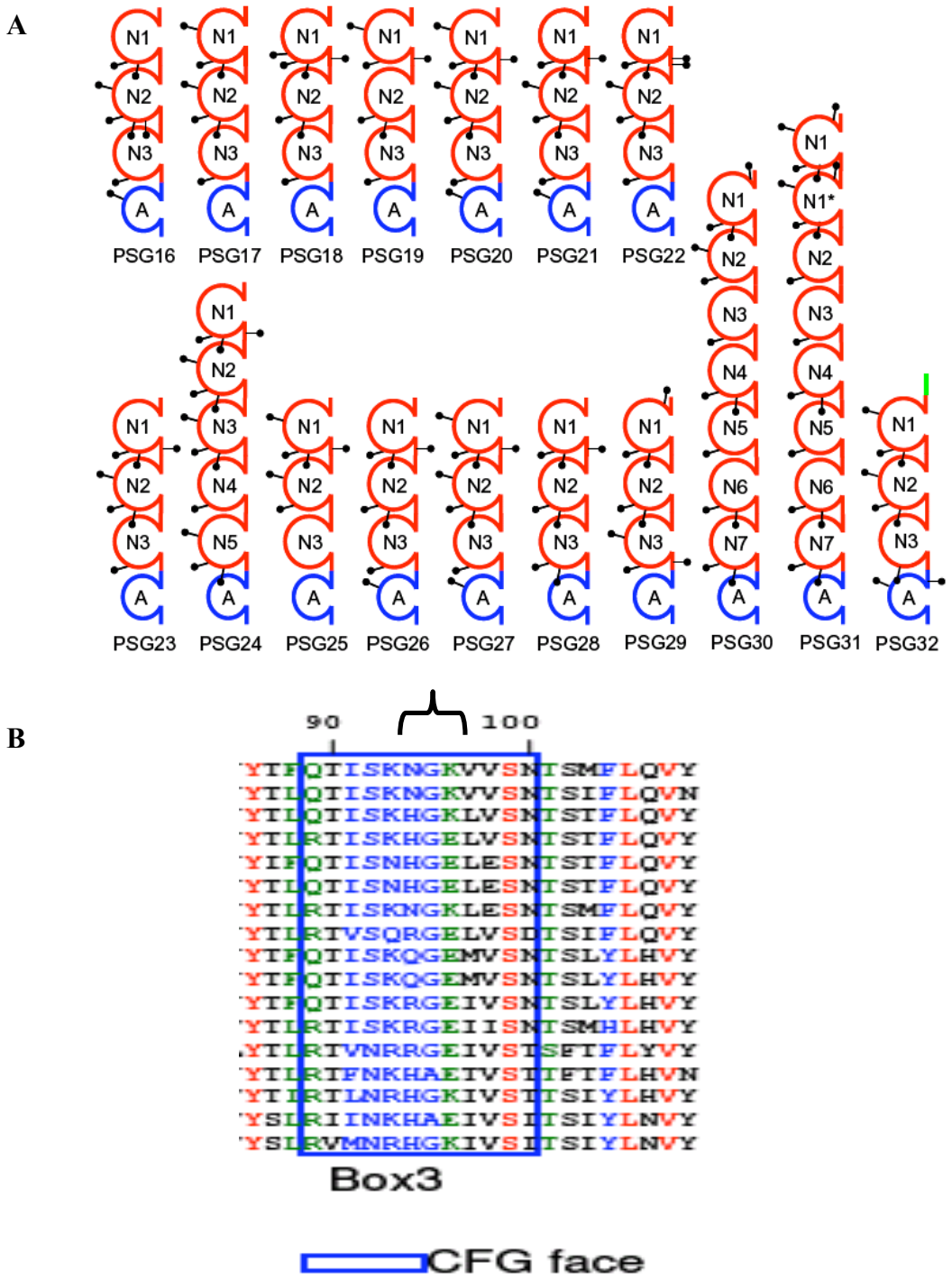

Figure 5. Mouse PSGs domain organization

(a) Mouse PSGs are composed of 3-8 IgV-like N domains and one IgC-like A domain [36]. **(b)** The murine N1-terminal domain corresponds to the human N-terminal domain. The RGD motif is not present in murine PSGs; however RGD-like motifs (RGE, HGE, and HAE) are aligned in a similar fashion on the solvent exposed loop of the N1 domain [37].

Transcripts for murine PSG17, 18, and 19 can be detected by reverse transcriptase (RT)-PCR in mouse embryonic and placental tissue, but not in adult tissues, including the ovary, testis, lung, kidney, spleen heart, thymus, brain, or liver. Immunofluorescence revealed PSG staining in the maternal decidua lining the capillaries within the implantation site [43]. Kromer et al. showed maximal PSG expression during placental development between embryonic day (E) 15.5 and E17.5 [51]. PSG protein and mRNA are detectable, correspondingly, in trophoblast giant cells from E8-E11 and the spongiotrophoblast from E14-E16 [43]. Wessells and colleagues were able to show placental PSG expression as early as 6.5 days *post coitum* in primary trophoblast giant cells as well [17].

4.2 Murine PSG function

As in humans, mouse PSGs have been shown to induce anti-inflammatory cytokines in macrophages indicating a similarity in pregnancy maintenance through maternal immune modulation. The N1 domain of these proteins is sufficient to elicit biological activity [15, 17]. Studies using a truncated version of PSG18 containing only the N1 domain (PSG18N) successfully upregulated IL-10 expression in murine macrophages [17]. Furthermore, PSG17N induced the secretion of IL-10, IL-6, TGF-β_1, and prostaglandin E_2 (PGE_2). In either case, PSG treatment did not result in the induction of IL-12 [15], IL-1β, TNF-α, or iNOS [17].

Previously, the Dveksler laboratory identified the receptor for PSG17 to be the tetraspanin CD9 [6]. To date, this is the only known PSG receptor and is not utilized by the human PSGs. Moreover, this specific receptor-ligand interaction is required for the aforementioned PSG17-mediated cytokine induction [15]. Additionally, CD9 was found

to be the receptor for another mouse PSG, PSG19[1]. These findings suggest CD9 (or another tetraspanin) may be required to mediate the biological activity of the other murine PSGs.

5 The Tetraspanin Family

5.1 Tetraspanins: A brief overview

The tetraspanin family was described in the early 1990s and to date consists of 33 members, including CD9, CD37, CD53, CD63, CD81, CD82, and CD151, to name a few. Initially discovered on surface of human leucocytes, tetraspanins prove to be conserved in species from *Schistosoma* to humans, and abundantly and widely distributed in all cells, with the exception of erythrocytes [52-55].

Members of the tetraspanin family exhibit significant sequence identity and common structural features. As a family, tetraspanins are classified as small, low molecular weight, membrane proteins. They posses a characteristic structure comprised of four highly conserved hydrophobic transmembrane domains, thereby forming two less conserved extracellular loops of unequal sizes and three relatively short cytoplasmic regions, including a small intracellular loop and amino and carboxyl tails. The more variable extracellular domains display variation in length, residue arrangement, and glycosylation sites. The small extracellular loop, EC1, comprises 20–28 amino acids and a large extracellular loop, EC2, contains 76–131 amino acids. (Figure 6) An important difference between tetraspanins is the number of cysteines in the EC2 [52-55].

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[1] Ha, C. et al, unpublished data

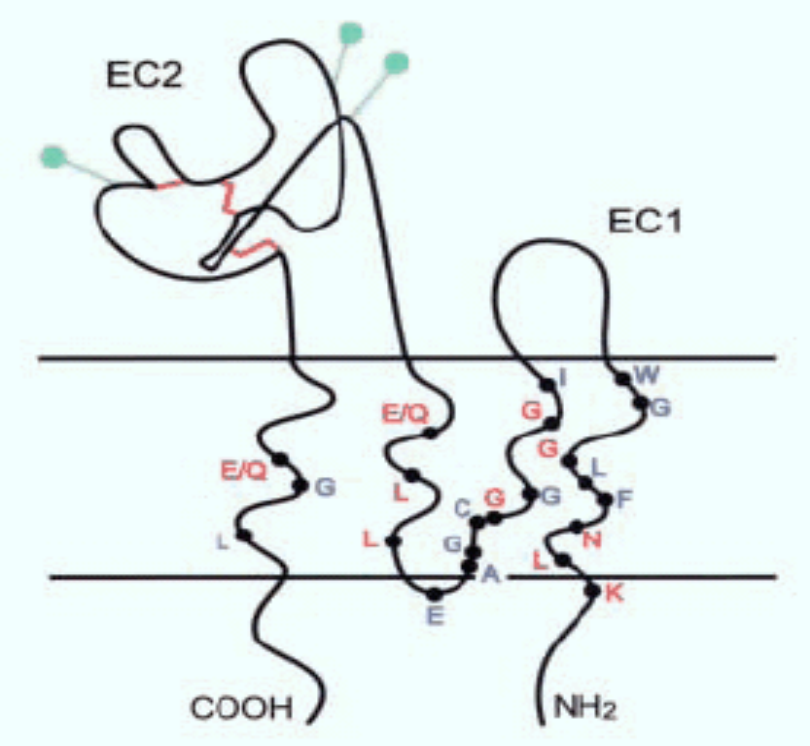

Figure 6. Basic Tetraspanin Structure

Generally, tetraspanins posses four hydrophobic transmembrane domains, creating two extracellular loops, one small and one large, and three short cytoplasmic regions, including a small intracellular loop and amino and carboxyl tails [53].

Tetraspanins have been implicated in a vast array of physiological processes, including signal transduction, immune cell activation, cell migration, cell–cell fusion (including fertilization), and differentiation. Also, studies suggest the importance of these molecules in various pathological outcomes, such as infectious disease [52-55], X-linked mental retardation, and metastasis [56]. However, the molecular mechanism through which tetraspanins act has not been fully elucidated.

A fundamental role of tetraspanins appears to be associating with various surface molecules and other tetraspanins forming a network of molecular interactions, known as the tetraspanin web [56-58]. Upon formation of the tetraspanin web, tetraspanins either act as a bridge or barricade, linking or separating, cell-surface molecules and their respective signaling partners, thereby mediating numerous physiological processes [58]. The tetraspanin web is structured in several different levels, with the tetraspanin-tetraspanin transmembrane interactions critical to its formation [56-58]. The first level involves detergent-resistant (i.e. Triton-X) primary interactions between specific tetraspanins and other proteins. The second and third level interactions are indirect and maintained only in weak detergents. Soluble second level complexes arise as tetraspanins associate with each other, subsequently linking multiple primary complexes [59]. Tetraspanin-associated proteins include integrins, Ig superfamily members, proteoglycans, complement-regulatory proteins, growth factor receptors and ligands, etcetera [60].

5.2 CD9

CD9 is a 24–27 kDa cell surface glycoprotein, originally identified as a surface antigen on lymphohematopoietic cells. CD9 is expressed by various cell types, including

epidermal, basophil, pre-B cells, activated T cells, platelets, neural cell lines [61], tumor cells, macrophages [62], and extravillous trophoblast. Extravillous trophoblast are responsible for invasion of the endometrium, proposing a role for CD9 during embryonic development [63].

The exact biological role of CD9 is unknown, recent studies showed that anti-CD9 mAb induced proliferation, adhesion and migration of Schwann cells [63, 64] and activated T cells independent of CD28 [65]. CD9 has been shown to associate with several combinations of β1 integrin complexes and increased CD9 expression can enhance integrin dependent B cell motility [61]. Additionally, CD9 is essential for gamete fusion during fertilization. Sperm-egg fusion is entirely inhibited in eggs from CD9-deficient females, rendering the homozygous mutant females infertile [66].

5.3 CD151

CD151, also known as SFA-1 and PETA-3 [67, 68], was initially identified as a marker of human acute myeloid leukemia cells, platelets, and vascular endothelial cells [69, 70]. The tetraspanin CD151 is expressed on DCs [71, 72], decidual NK (dNK) cells [73], activated T lymphocytes [68], erythrocytes [74], epithelial cells, endothelium, platelets, muscle cells, and megakaryocytes [67-69]. (Figure 7) The hematopoietic cell lines M07e, HEL, and K562 display both intracellular and extracellular expression of CD151 [68]. Up to 66% of CD151 resides in an intracellular endosomal/lysosomal compartment in cultured human umbilical vein endothelial cells (HUVECs) [67].

The human CD151 gene is located on chromosome 11p15.5 and encodes a 253-amino acid CD151 protein. CD151 is structurally a typical tetraspanin, with a single N-glycosylation site on its large extracellular loop and is palmitoylated on several cysteine

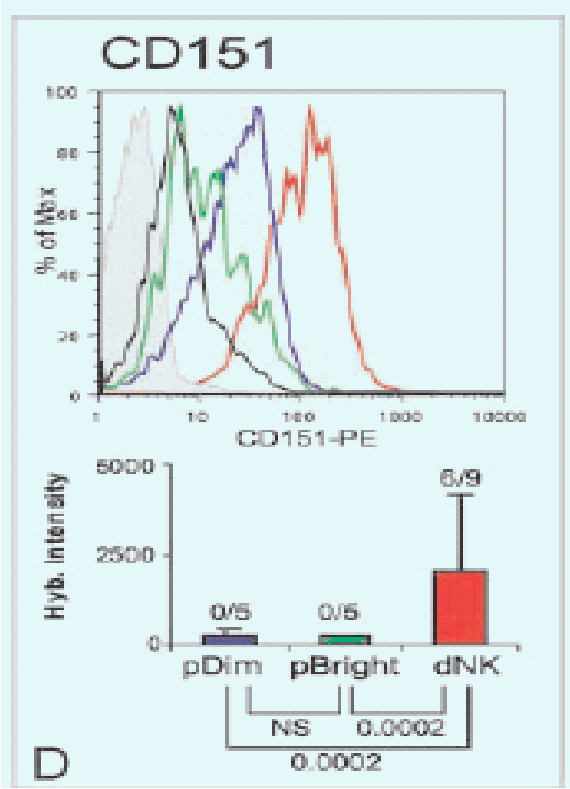

Figure 7. CD151 is overexpressed in dNK cells

FACS analysis reveals overexpression of CD151 in decidual NK cells (red) compared to CD56 ^{bright} (green) and CD56 ^{dim} (blue) peripheral NK cell. The elevated expression levels may indicate a potential role for CD151 in normal pregnancy maintenance [73].

residues. The unglycosylated and glycosylated forms are 28 and 32 kDa, respectively [74].

Membrane-bound CD151 has been shown to associate with other members of the tetraspanin family [68, 74], namely CD9, CD63 and CD81 [68] as well as, β1 integrins,α3, α6, and α7 chains, and α6β4 complexes [75]. Integrins are a major family of cell surface receptors for extracellular matrix proteins. Integrin-mediated cell adhesion directs cell migration, differentiation, signaling, and cytoskeletal organization, among other functions. Tetraspanins, through their association with integrins, modulate cell migration, fusion, and signaling, but not integrin-dependant cell adhesion [67, 76]. Unlike other tetraspanin-integrin associations, CD151 forms a stable, Triton-X-resistant, complex with α3β1, α6β1, and α7β1 [74]. The integrin α3β1 links to the EC2 domain of CD151 between the Leu^{149} and Glu^{213} residues [76, 77]. It is proposed that CD151 is always coupled to integrins, and this association, might be a requirement for α3β1 integrin maturation and cell surface expression [74, 76]. CD151 also interacts with PI4-K and PKC, thereby linking integrin α3β1 to these signaling molecules [76].

Functional assays indicate a role for CD151 in cell-cell adhesion, cell migration, platelet aggregation, neovascularization, and angiogenesis [70]. On endothelial and epithelial cells, CD151 localizes to cell–cell junctions and modulates cell migration and invasion [67]. Additionally, CD151 may mediate its migratory and angiogenic effects during pregnancy, evidenced by its expression in human placenta [76]. Moreover, overexpression of CD151 (and CD9) in dNK cells suggests a correlation to the mechanisms used by dNK cells for migration to the pregnant uterine mucosa. [73].

Wright et al. generated CD151-null mice demonstrating no signs of lethality. These mice are typically healthy and fertile; however, the CD151-null mice do show an abnormal hemostasis and CD151-null keratinocytes display poor migration [70]. The absence of severe phenotypes in CD151-null mice could result from functional compensation by other tetraspanins [76].

6 Embryogenesis and Placentation

There are many important factors that come into play to ensure proper fetal growth and development. The commencement of human development manifests as haploid paternal and maternal gametes join forming a diploid zygote in a process known as fertilization [78]. (Figure 8) Fertilization occurs in the fallopian tube within 24 to 48 hours of ovulation [79], after which the zygote undergoes several rounds of division, developing into a blastocyst, comprising an inner and outer cell mass. The inner cell mass gives rise to the embryo proper and the surrounding trophectoderm differentiates into a variety of trophoblast cell subtypes with different functions. Trophoblast are embryonic cells responsible for developing into the placenta, providing nourishment from the mother to the fetus, and implantation [80]. (Figure 9)

Embryonic implantation is initiated by the interaction between the blastocyst and maternal uterine luminal epithelium [81], requiring both prior preparation of the receptive uterus and activation of the blastocyst [82, 83]. The endometrium undergoes a series of changes, mediated primarily by estrogen and progesterone [83], leading to a period of receptivity; outside of this window the uterus is resistant to embryonic attachment [84].

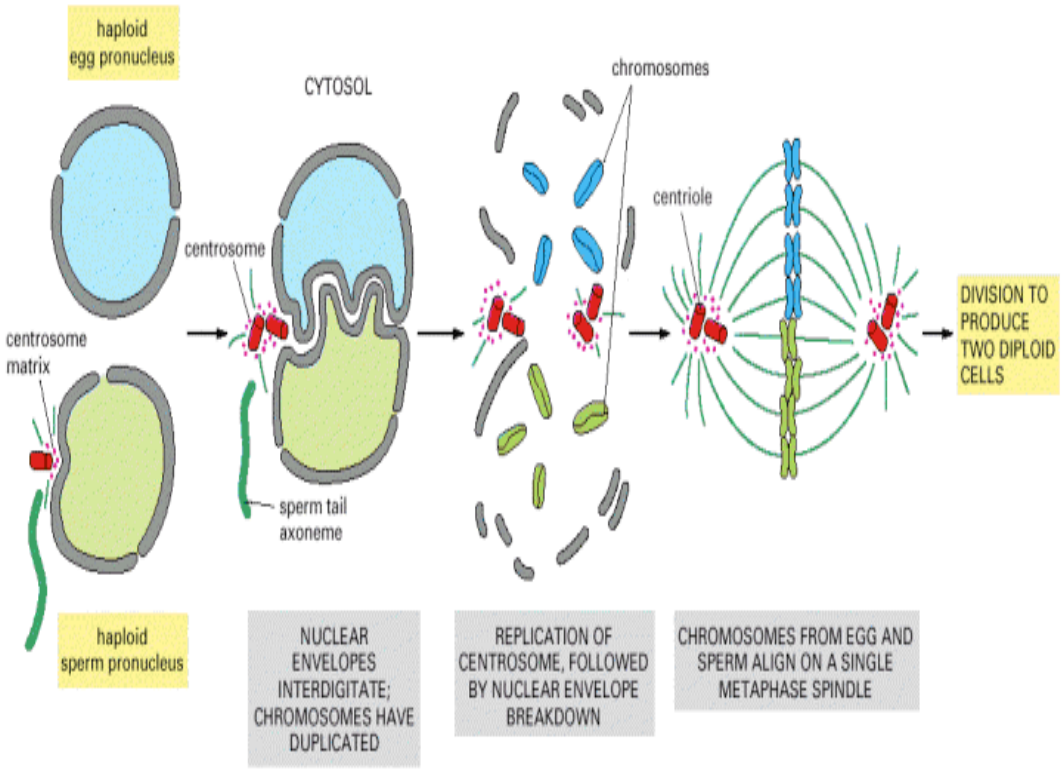

Figure 8. Gamete fusion during Mammalian Fertilization

Haploid paternal and maternal gametes join forming a diploid zygote. The sperm centriole enters the egg along with the sperm nucleus and tail. It replicates, the nuclear envelopes break down, and the chromosomes of both gametes are integrated into a diploid zygote [85].

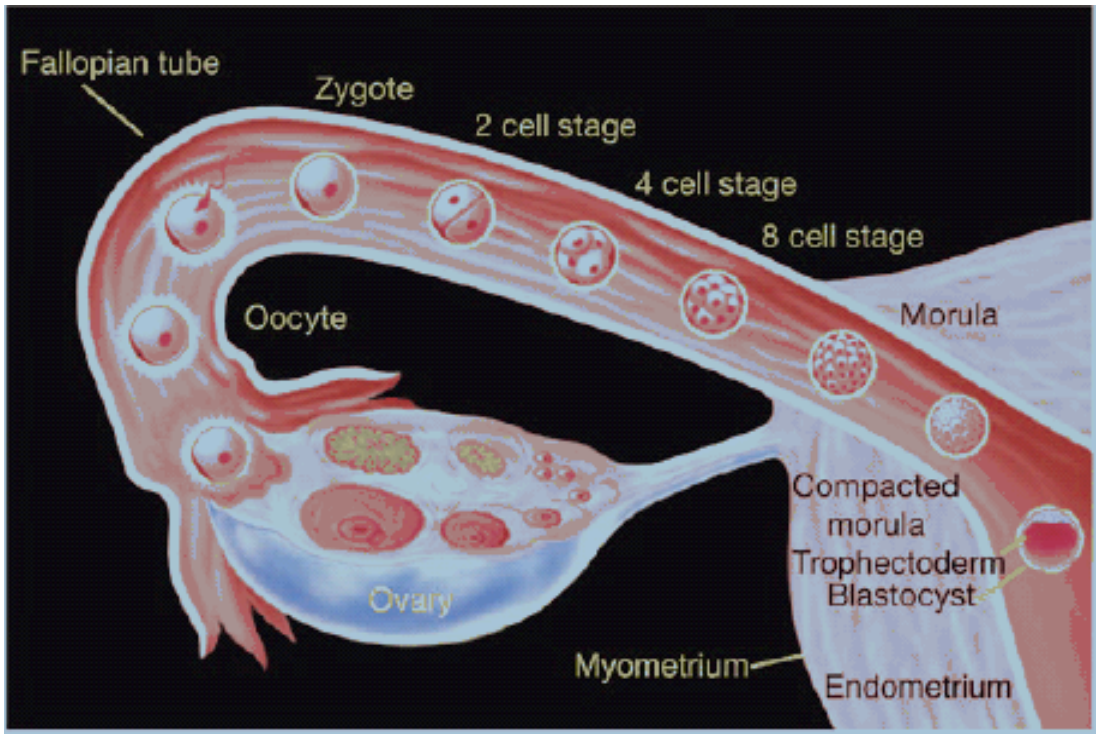

Figure 9. Key pre-implantation events in mammalian development

After fertilization the ovum undergoes several rounds of cleavage, forming a blastocyst. The Blastocyst consists of an inner and outer cell mass. The inner cell mass gives rise to the embryo and the outer cell trophectoderm is responsible placental development [79].

Once the blastocyst hatches from the zona pellucida, approximately 6 days after fertilization [79], trophoblasts are attracted to, migrate towards, and invade the decidua and myometrium [86]. At around gestational day 8, the stromal cells surrounding the implanting blastocyst undergo decidualization, eventually embedding the embryo in the antimesometrial stromal bed [82]. Embryogenesis continues for a total of 8 weeks, followed by further extensive development of fetal tissues and organs until parturition [87]. During the first few weeks of pregnancy, the villous structure of the placenta develops; together the decidual layer and select trophoblasts form the placenta [82]. (Figure 10) Achievement of proper uteroplacental circulation and fetoplacental vasculature requires trophoblast mediated maternal spiral arteriole transformation [88].

6.1 Haemochorial Placenta

The chorioallantoic placenta is a unique structure to eutherian mammalian development and is the 1st organ to develop during mammalian embryogenesis [89]. The placenta can be grouped histologically, based on the degree of separation of maternal and fetal blood, into three categories, i.e. epitheliochorial, endotheliochorial and haemochorial [90-92]. (Table 1) The haemochorial placenta is present in various orders [91] including primates and rodents [93]. As with everything, this type of placentation has its pros and cons; while the close connection between maternal and fetal tissues proves advantageous in facilitating direct access to maternal blood for oxygen–carbon dioxide exchanges, doing so leaves the fetus at an increased risk of maternal immune rejection [91, 93].

The placenta and fetal membranes function physiologically to form a barrier against infection and maternal immune attack and transport essential nutrients and waste

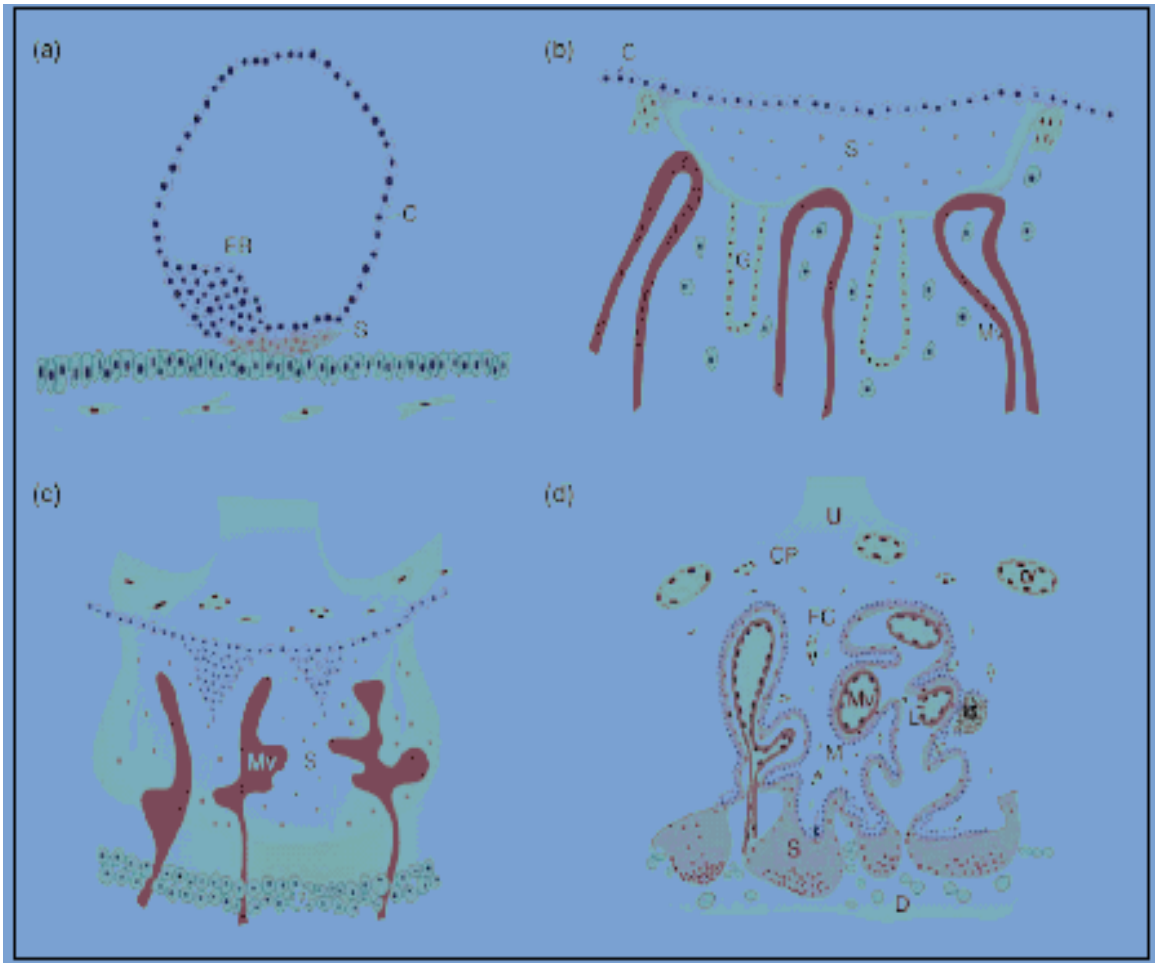

Figure 10. Early haemochorial placental development

(a) Prior to embryonic implantation the blastocyst trophoblast cells differentiate into subgroups: Syncytiotrophoblast (S) & Cytotrophoblast (C)

(b) The syncytiotrophoblast are the fetal cells that form the villous surface of the placenta

(c) After penetrating maternal tissue, lacunae develop within the syncytiotrophoblast, cytotrophoblastic cones grow, and fetal capillaries begin to appear within the chorion plate.

(d) Trophoblast branching occurs, resulting in a trabecular tree, comprising mesenchyme (M), fetal capillaries and a cytotrophoblasts layer. Syncytiotrophoblast form a barrier to the labyrinth (L). Maternal vessels (Mv) are opened allowing the placental labyrinth to be filled with maternal blood, thereby forming the haemochorial placenta [94]

Placenta type	Superorder	Order
Haemochorial	Laurasiatheria	Carnivora—hyaenas only
		Chiroptera—many bat families
		Insectivora—hedgehogs
	Euarchontoglires	Rodentia—most families
		Lagomorpha—rabbits, pikas
		Primata—monkeys, gorillas, man, Tarsius
		Dermoptera—flying lemurs
	Xenarthra	Xenarthra—armadillos, anteaters
	Afrotheria	Hyracoidea—hyraxes, conies
		Afrosoricida—tenrecs, golden moles
		Macroscelidea—elephant shrews
Endotheliochorial	Laurasiatheria	Carnivora—all but hyaena
		Pinnipedia—seals, walruses
		Chiroptera—several bat families
		Insectivora—shrews
	Euarchontoglires	Rodentia—kangaroo rat
		Scandentia—tree shrews
	Xenarthra	Xenarthra—sloths
	Afrotheria	Proboscidea—elephants
		Tubulidentata—aardvark
		Sirenia—manatee
Epitheliochorial	Laurasiatheria	Cetacea—whales, porpoises
		Artiodactyla—cows, pigs, deer
		Perissodactyla—horses, tapirs
		Pholidota—pangolin
	Euarchontoglires	Primata—lemurs, lorises

Table 1. Placental types in Eutherian Mammals

There are three placental categories, haemochorial, as seen in humans, endotheliochorial, and epitheliochorial [91].

products to and from the fetus. In addition, the placenta is an important source of pregnancy associated hormones and growth factors [26, 89]. Hormones shown to inhibit lymphocyte reactivity are produced in high concentration at the fetal maternal interface including progesterone, human placental lactogen (hPL), prolactin, and estrogens [95]. Other immune regulating factors include human chorionic gonadotropin (hCG) [96], pregnancy specific glycoprotein, pregnancy-associated plasma protein A, α-fetoprotein, as well as an array of cytokines, including IL-6, and TGF-β [95]. Dysregulation in placental formation and/or function can result in early pregnancy loss or pregnancy complications. Immune dysfunction is becoming more apparent as a prominent cause for placenta-associated disorders [89].

7 Immune Tolerance at the Fetal Maternal Interface

Normal pregnancy is characterized by a decrease in cell-mediated anti-fetal immunity and a dominant humoral immune response [97]. During pregnancy, the embryo/fetus, containing paternal antigens, is viewed by the maternal immune system as a semi-allogeneic graft; however, in an uncompromised state, the maternal immune system allows the fetus to develop within the uterus until parturition. The mechanism of immune privilege and tolerance at the fetal-maternal interface remains an immunologic paradox [5]. It is clear however, that the fetus survives as a result of cooperative interactions between the fetus and the mother throughout pregnancy [98]. Both fetal and maternal organs and tissues, populated mainly by cells of the innate immune system, contribute to fetal tolerance by producing hormones, prostaglandins, cytokines, and chemokines that promote fetal immune privilege [5].

7.1 Trophoblasts and immune regulation

Trophoblasts are essential for the modification of the maternal uterine environment into a hospitable site for embryonic and fetal development [99]. During pregnancy trophoblasts are responsible for promoting the switch from acquired to innate immunity by manufacturing chemokines that attract cells of the innate immune system into the uterus. Also, they are a significant source of soluble immuno-suppressants, such as progesterone and prostaglandins, human leukocyte antigen G (HLA-G), TGF-β_1, IL-10 [98], and indoleamine 2,3-dioxygenase (IDO) [30].

7.1.1 HLA-G

HLA-G is a nonclassical major histocompatibility complex (MHC) class I antigen expressed in the placenta, almost exclusively by the invasive extravillous cytotrophoblasts [100-103]. HLA-G protein and mRNA levels are reduced when trophoblast invasion is defective and shallow, as it is in pre-eclampsia, suggesting that HLA-G expression is directly related to the level of invasiveness displayed by the extravillous CTB [103]. This nonclassical class I antigen is thought to play an essential role during pregnancy by protecting the semi-allogeneic fetus from recognition and destruction by maternal immune cells. It acts as a ligand for inhibitory receptors present on NK cells, T cells, and macrophages, rendering them incapable of mounting an immune attack on the fetus [100-103]. Similarly, HLA-G transgenic mice are more tolerant to skin allografts, compared to control mice, due to HLA-G mediated inhibition of DC maturation and their subsequent inability to receive signals from activated T cells [104].

7.1.2 IDO

Indoleamine 2,3-dioxygenase is an enzyme that degrades the essential amino acid tryptophan, resulting in G1-arrest of activated T-lymphocytes, thereby promoting immune tolerance [105]. During pregnancy, the cells that produce IDO are located at the maternal–fetal interface in human term placenta and in the maternal decidua following implantation in pregnant mice [106]. More specifically, expression has been observed in placental STB and endothelial cells, as well as invasive extravillous trophoblast in early and term deciduas. Since the extravillous trophoblast are in closest contact with the maternal immune system, Hönig et al. suggest they protect the fetus from rejection by downregulating local maternal T-cell responses [105, 107]. In fact, IDO inhibition in mice causes a maternal T-cell reaction against paternal antigens, ultimately culminating in fetal demise [108, 109].

7.2 Th1/Th2 balance

It is common knowledge that proper pregnancy maintenance requires adequate function of the cytokine network [110]. Th1 and Th2 cells are the major subsets of differentiated T helper cells, with distinct functional properties that correlate with their characteristic cytokine profiles. Th1 cells generally arbitrate cell-mediated inflammatory reactions, whereas Th2 cells are engaged mainly in the regulation of the humoral response [110, 111].

Traditionally, Th1 cytokines are viewed as the “bad boys” of pregnancy [112]. Studies implicate Th1 cytokines as detrimental; TNF-α and IFN-γ are responsible for trophoblast killing and inhibition of both mouse embryonic implantation and trophoblast proliferation [111]. Conversely, Th2 cytokines, including IL-10, appear to play a protective role [112], as reduced Th2 activity has been linked to RSA [113]. Moreover,

administration of IL-10 was shown to decrease the rate of fetal resorption in abortion-prone mice [114]. Another noteworthy finding is the exacerbation of lupus erythematosus (Th2 dependent) and diminution of rheumatoid arthritis (Th1 dependent) during human pregnancy [110].

Consequently, more than a decade ago, Wegmann and colleagues postulated the "immunotrophic hypothesis" suggesting that pregnancy success is dependent on Th2 dominance with a concomitant suppression of Th1 activity. However, we now know that Th1 cytokines are essential for a prosperous pregnancy as well. They have vital roles during implantation, by promotion of a Th2 response, and initiation of term delivery. Therefore, a coordinated cooperation of these cytokines, rather than dominance, seems to be an important component of the immunological reactions during pregnancy [110]. The predominant cytokines at each gestational stage function to restrict maternal immune rejection and to facilitate the on-going physiological processes within the maternal reproductive tract [113]. (Figure 11)

8 TGF-β, IL-10, and IL-6 in Pregnancy

8.1 Transforming Growth Factor β

The transforming growth factor-beta (TGF-β) superfamily are secreted signaling molecules with multifaceted immunoregulatory properties [115]. The TGF-β superfamily consists of a large group of growth and differentiation factors, such as TGF-β isoforms, activins, inhibins, growth and differentiation factors (GDFs), and bone morphogenetic proteins (BMPs) [116]. As a family they regulate a wide range of cellular processes, such as growth, differentiation, apoptosis, morphogenesis [117], angiogenesis, decidualization, and implantation [118].They function through serine/threonine kinase type I and II cell

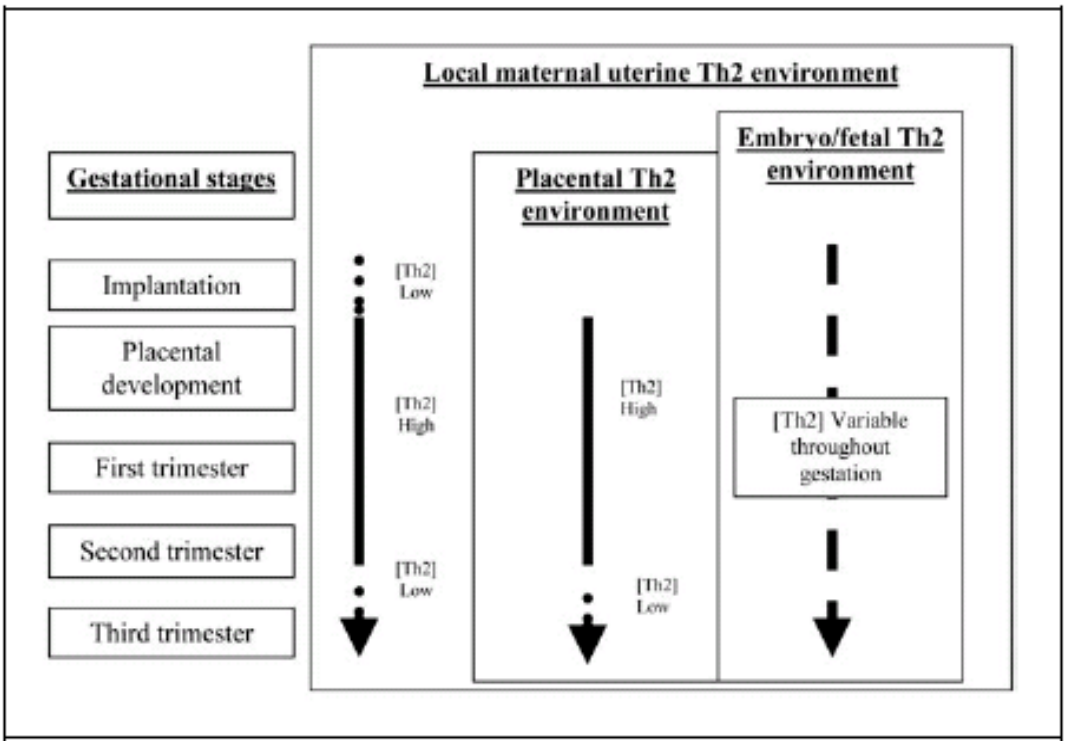

Figure 11. Th1/Th2 balance during pregnancy

Successful pregnancy is, in part, a result of the coordinated cooperation of the cytokine network. The Th1 cytokines seem to predominate during the early implantation stages and just prior to parturition. Whereas elevated Th2 cytokine levels are required during the core of pregnancy, probably since they appear to have fetal protective roles [113].

surface receptors that subsequently activate Smad proteins, which relocate to the nucleus and activate target gene transcription. Mutations in these pathways or abnormal ligand expression or function are implicated in numerous pathological conditions [116, 118, 119].

Early in pregnancy, TGF-βs appear to be involved in tissue remodeling, immune cell regulation, formation of extracellular-matrix, and angiogenesis [120]. It is required for embryonic implantation and is involved in regulating decidual [117] and placental development and functions [116, 117]. (Figure 12) Studies show a significant reduction in TGF-β levels in the uterine epithelial and metrial gland NK cells of mice prone to a pregnancy failure.

During pregnancy, all three isoforms, TGF-β1, 2, and 3, can be found in the amniotic fluid [117] and decidua, with TGF-β_2 primarily localized to stroma while TGF-β_1 and TGF-β_3 are present in epithelial cells as well as stromal cells [121]. During peri-implantation, both TGF-β receptors I and II play important roles in uterine receptivity and blastocyst attachment [118]. Additionally, TGF-β_1 may function as a regulatory factor in the protection of fetal rejection [117, 121]. It plays a central role in the peripheral immune system by attaining immune tolerance, thus facilitating embryonic implantation and development. This notion is evidenced by its ability to inhibit T helper type 1 (Th1) responses, thereby downregulating IFN-γ and inflammatory cytokine production [121]. *In vitro* studies link TNF-α and IFN-γ to the inhibition of mouse embryo implantation and human trophoblast proliferation [111].

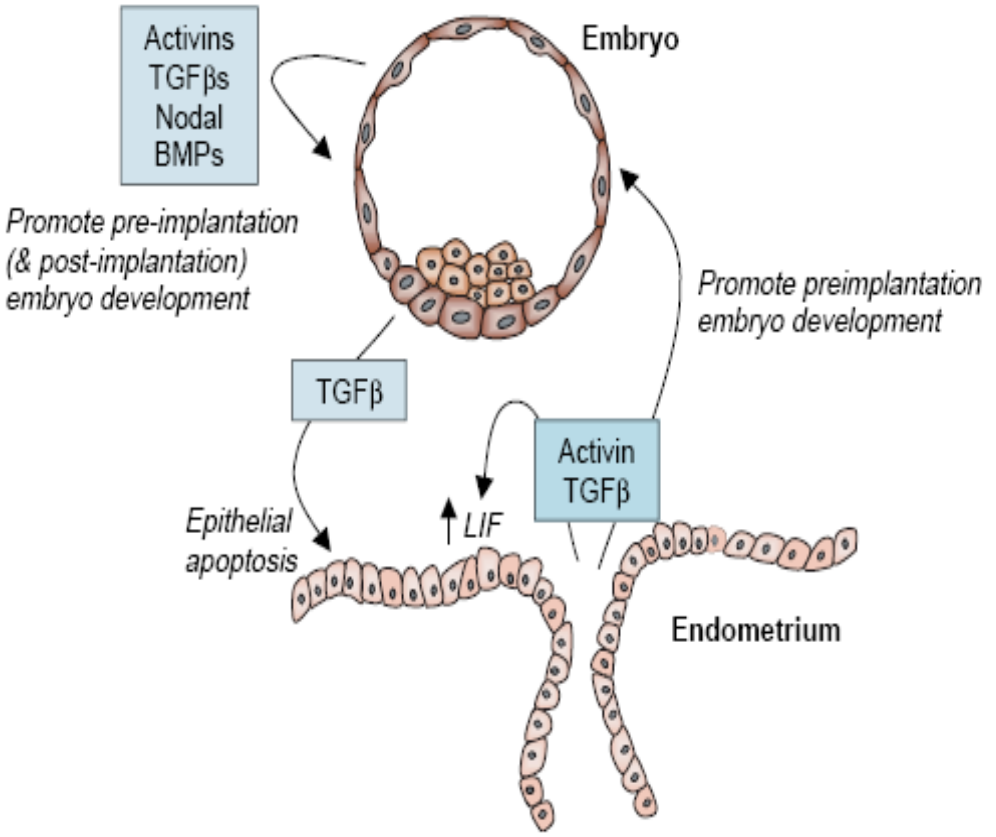

Figure 12. TGF-β superfamily during embryonic implantation

Activins and TGF-βs are produced and secreted by the epithelial lining of the fallopian tube and uterus. Moreover, TGF-β receptors are expressed in the fallopian tube and uterine epithelial cells. *In vitro* studies, in addition to the aforementioned, suggest a role in pre- and post-implantation embryo development and differentiation. During implantation, blastocyst secreted TGF-β has been implicated in the preparation of the endometrium for implantation through endometrial epithelial cells apoptosis [121].

8.2 Interleukin 10

IL-10 is a potent immune-regulating cytokine that inhibits inflammatory cytokine synthesis [122]. It is produced predominately by T cells, both Th1 and Th2 cells, as well activated monocytes/macrophages, B lymphocytes, and dendritic cells in response to LPS stimulation. Indirectly, through the downregulation of APCs, IL-10 inhibits the secretion of Th1-derived cytokines. In addition, IL-10 inhibits several macrophage functions, such as oxidative burst, phagocytosis, and nitric oxide production [123, 124]. IL-10 serves as an excellent negative feedback regulator [125]; it operates by deactivating macrophages, silencing their synthesis of pro-inflammatory cytokines and chemokines [95, 125], and returning the body to its resting state [125].

During pregnancy, progesterone, catecholamines, prostaglandins [126], and trophoblasts [95] have been shown to induce IL-10 production as well. IL-10 is expressed abundantly in a gestational stage-dependent manner in the maternal uterine decidua and placental tissues. Maximum expression occurs during the first two trimesters and attenuates during the third trimester. This may be in part, due to its inhibitory effect on matrix metalloproteinase-9 production, as MMP-9 is induced in response to labor by villous trophoblasts [126].

Murphy et al. implicate IL-10 in protection of the fetus against uNK cell inflammatory demise [127]. *In vitro* studies demonstrate the ability of IL-10 to downregulate synthesis of TNF-α in human chorion, decidual, and placental cells. Furthermore, administration of recombinant IL-10 was able to successfully rescue LPS induced fetal loss in IL-10 null mice [122]. In fact, in another mouse study, injections with an anti-IL-10 monoclonal antibody resulted in a prominent increase in the rate of

fetal loss [128]. In rats, administration of exogenous IL-10 can alleviate LPS induced fetal loss and growth restriction as well as abrogate premature birth elicited by Escherichia coli uterine infections [122]. Moreover, exposure of LPS-treated macrophages to IL-10 prior to co-culture prevented their ability to inhibit trophoblast invasion [129].

Abundance of endogenous IL-10 in gestational tissues is key in preterm labor resistance. [122]. IL-10, prevents fetal wastage in mice prone to fetal resorptions and, conversely, the administration of anti-IL-10 antibodies increases fetal wastage [111]. Studies suggest that elevated IL-10 is a physiological consequence of normal healthy pregnancy, with higher IL-10 production in successful pregnancy when compared to spontaneous abortion [130]. In fact, women who suffer from recurrent miscarriages are less likely to have IL-10 positive decidual T-lymphocytes. Additionally, pre-eclampsia is associated with reduced maternal levels of immunoregulatory cytokines, such as IL-10 [129], thereby implicating IL-10 as a potential therapeutic agent [122].

8.3 Interleukin 6

IL-6 is a diverse cytokine functional in both innate and adaptive immune responses [125]. It is produced by a wide array of cells including APCs, fibroblasts, endothelial cells, neuronal cells, macrophages, mast cells, and CD4+ Th2 cells [131]. In innate immunity, IL-6 contributes to the systemic effects of inflammation by stimulating the synthesis of acute-phase proteins; however, in adaptive immunity it stimulates the growth of differentiated B lymphocytes [125]. Additionally, IL-6, in the presence of IL-4, promotes the differentiation of CD4+ T cells into Th2 cells while inhibiting the production of Th1 cells [131-133]. (Figure 13)

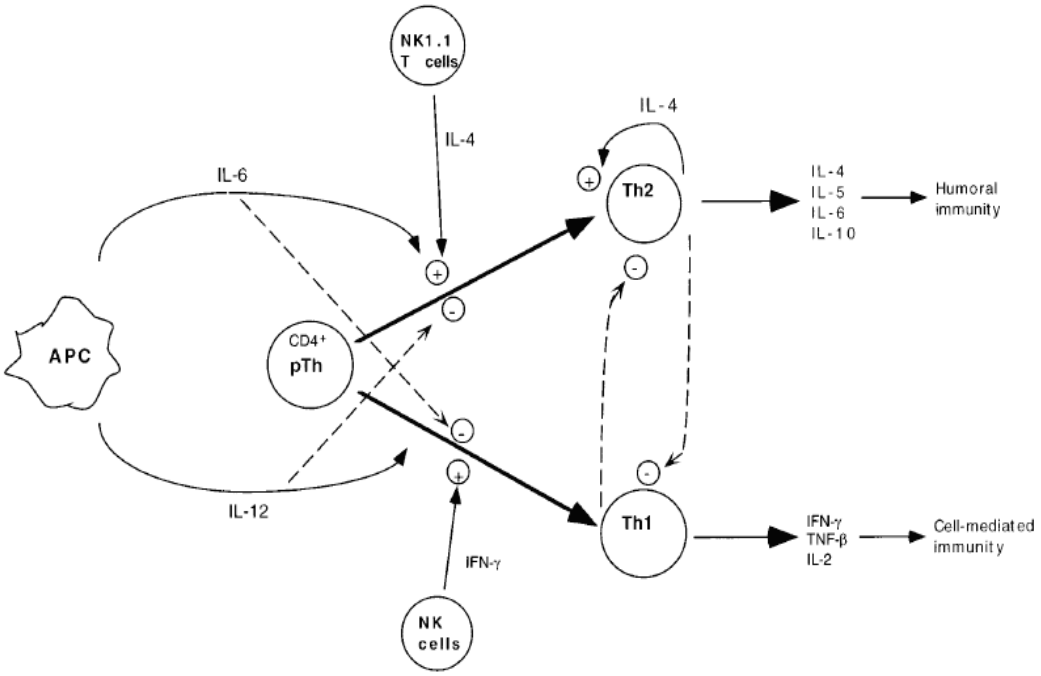

Figure 13. IL-6 promotes the differentiation of CD4+ cells into Th2 cells

IL-4 and IL-12 are the major players in the differentiation of naive CD4+ T cells to the Th2 and Th1 phenotype, respectively. APC-secreted IL-6 induces IL-4 production and together, with IL-4, directs the differentiation of CD4+ cells into Th2 cells while inhibiting the IL-12-mediated generation of Th1 cells [131].

IL-6 is often grouped together with pro-inflammatory cytokines TNF-α and IL-1 [134], however, IL-6 has been shown to inhibit TNF production both *in vivo* and *in vitro*. Studies show that IL-6-deficient mice have increased TNF levels when injected with LPS, implicating endogenous IL-6 in downregulating TNF production [135] without compromising the level of anti-inflammatory cytokines [134]. Furthermore, Turner et al. demonstrate the induction of IL-6 in human peripheral blood mononuclear cells treated with TGF-β_1. The ability of TGF-β_1 to induce IL-6 suggests they may mediate similar effects [136].

During pregnancy, the STB cells of the placenta abundantly secrete IL-6 [137], which subsequently stimulates the release of hCG and hPL [138], suggesting a possible immunoregulatory role for IL-6 at the fetal-maternal interface [137]. Li et al. suggest that trophoblast-IL-l stimulates trophoblast production of IL-6 and activates an IL-6 receptor-mediated pathway, thus releasing hCG from trophoblasts [139]. Also, elevated IL-6 mRNA expression has been observed in stimulated ovaries and mouse embryonic implantation sites, suggesting IL-6 plays a role in angiogenesis, specifically during folliculogenesis and maternal decidual formation [140].

9 Arginase, Cyclooxygenase 2, and Prostaglandin E_2 in Pregnancy

9.1 Arginase

Mammalian arginase exists as two distinct isoenzymes, type I arginase (Arg1), a cytosolic hepatic enzyme of the urea cycle, while type II arginase (Arg2), a mitochondrial enzyme, is expressed at lower levels in kidney, brain, small intestine, mammary gland, and macrophages, with little or no expression in liver [141, 142]. A variety of agents,

including, IL-4 and IL-10, cAMP, TGF-β, PGE_2, LPS, and COX-2 have been shown to induce arginase in macrophages [48] and dendritic cells[141, 142].

Arginase is found in the uteri of nonpregnant and pregnant animals, and its activity is increased during pregnancy [143]. Arginase catalyzes the final step of the urea cycle; it competes with iNOS for L-arginine, producing L-ornithine and urea, rather than NO [48, 141, 142] and thereby reduces the risk of pre-eclampsia [48].

Arginase has been suggested to play an important role in cellular growth and development, particularly important to the fetus, by supplying L-ornithine for the synthesis of polyamines. Additionally, arginase is present in the myometrium of pregnant rats [144], with maximal activity at the implantation site, also in the liver, kidney, small intestine, and stomach. In fact, recombinant PSG1a induces upregulation of arginase activity with a concomitant downregulation of iNOS activity [48].

9.2 Cyclooxygenase 2

The enzyme cyclooxygenase, also known as prostaglandin endoperoxide synthase, exists in two isoforms, COX-1 and COX-2, which are responsible for the conversion of arachidonic acid (AA) to various prostaglandins (PG) [145-149]. (Figure 14) Prostaglandins play a vital role in inflammation, immune function, mitogenesis [148], and in several female reproductive functions such as ovulation, fertilization, establishment and maintenance of pregnancy, and parturition [150]. In many cases, different PGs will have opposing actions [151] that can contribute to various pathological outcomes, including Alzheimer's disease, cancer, and arthritis [147].

While structurally and kinetically similar, COX-1 and -2 encoded by two separate genes found on chromosome 9 and 1, respectively, show signs of distinct expression,

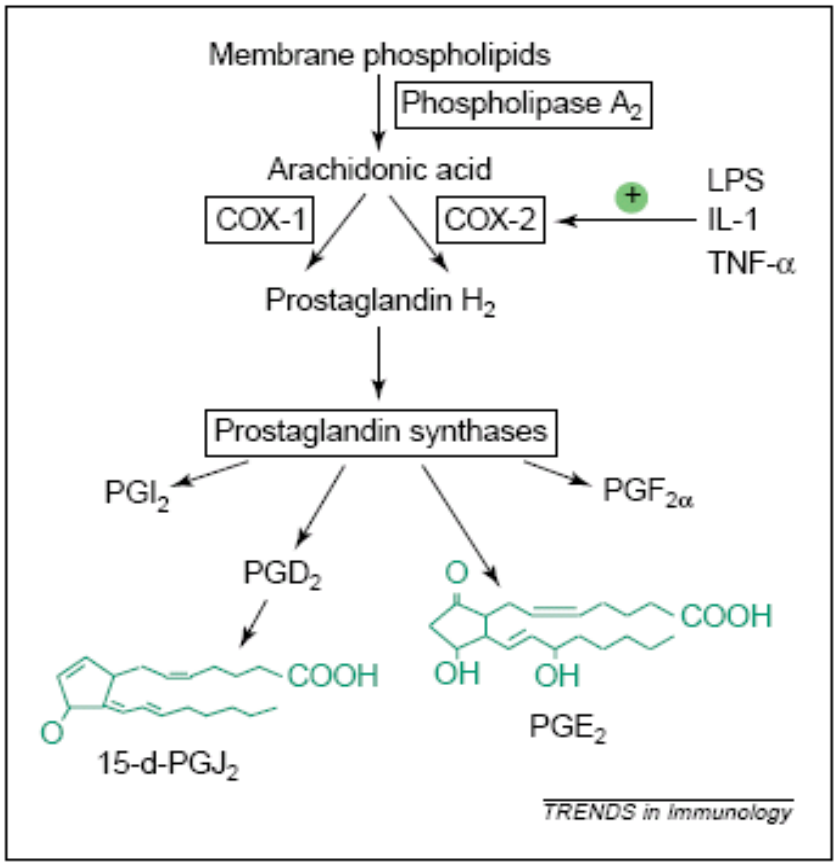

Figure 14. Prostaglandin synthesis

Phospholipase A_2 frees arachidonic acid from the membrane, making it available to COX-1 and COX-2, which subsequently produce PGH_2. PGH_2 is acted upon by prostaglandin synthases to produce a variety of prostanoids [152].

regulation, and subcellular localization [146, 148]. COX-1 is present and constitutively expressed in most cells and tissues [145, 146, 149, 153], whereas COX-2 is induced by a variety of cytokines, specific inflammatory stimuli, and growth factors [154], with basal levels detected in the brain, kidney and the gravid uterus [148]. Both isoenzymes metabolize arachidonic acid to prostaglandin H_2; however the subsequent conversion into distinct prostanoids depends on the catalyzing isoform. In resident peritoneal macrophages, COX-1 shifts PG production primarily to prostacyclin (PGI_2), thromboxane A_2 (TXA), PGD_2, and 12-hydroxyheptadecatrienoic acid (HHT). On the other hand, LPS-induced COX-2 results in the production of PGI_2 and PGE_2 [151].

During early pregnancy, COX-1 and COX-2 are expressed and may be important for implantation and angiogenesis. Production of COX-1 in the amnion is increased by hCG and therefore may be important in pregnancy maintenance [148]. COX-2, but not COX-1 null mice display defects in ovulation, fertilization, implantation, and decidualization [155].In fact, COX-2-defiecient mice are infertile due to reduced ovulation [148]. COX-2 is expressed in the syncytiotrophoblast of the chorionic villi [156] and the luminal epithelial and stromal cells surrounding the active blastocyst during the attachment reaction were it synthesizes PGI_2 [155] and PGE_2 essential for implantation and decidualization [157], as well as initiating uterine contractions during labor, evidenced by elevated COX-2 mRNA levels parturition [148].

9.3 Prostaglandin E_2

PGE_2 is a potent immunomodulator believed to suppress cell-mediated immunity while favoring the humoral immune response [158]. PGE_2 is produced by a range of cells including fibroblasts, macrophages and some malignant cells [152]. PGE_2 is responsible

for mediating varied biological processes, including cardiovascular, pulmonary, renal, endocrine, gastrointestinal, neural, reproductive, and immune function [157]. It modulates T cell, B cell, and APC activity through binding to one, or more, of its four subtypes of receptor (EP_1, EP_2, EP_3 and EP_4) [152]. They belong to the seven transmembrane family of receptors and exert their function via G-proteins [156]. Prostaglandin E_2 regulates the production of a wide array of cytokines; it stimulates a dramatic reduction in the synthesis of TNF-α and IL-12, while augmenting IL-6 and IL-10 levels [158, 159]. (Figure 15)

PGE_2 modulates the activities of APCs and T cells in a variety of ways. PGE_2 inhibits CD4+ T cell proliferation and induces immature T cell apoptosis, promoting anergy. In macrophages, PGE_2 acts to down-regulate the expression of the IL-12 receptor and inhibit pro-inflammatory cytokine production. It has divergent effects on DC activation; in peripheral tissues, PGE_2 induces DC activation, however, once in the lymphoid organs, PGE_2 inhibits the maturation of DCs and their ability to present antigen [152, 160]. Also, PGE_2 regulates DC cytokine production, promoting the differentiation of naive T cells into Th2 cells [152, 156, 160]. PGE_2 has been shown to play a role in regulating the Th1/Th2 balance. Because Thl and Th2 cytokines negatively cross-regulate each other's production, the selective inhibition of Thl cytokines could result in dominant Th2 response [161] beneficial during pregnancy [150].

PGE_2 serves as an immunomodulatory mediator at fetal-maternal interface, as a mitogenic, anti-apoptotic, and angiogenic factor, and either as a myometrial relaxant or stimulant. Moreover, increased PGE_2 expression correlates with gestational progression [150]. Among the PGs, PGE_2 is considered particularly important for implantation and

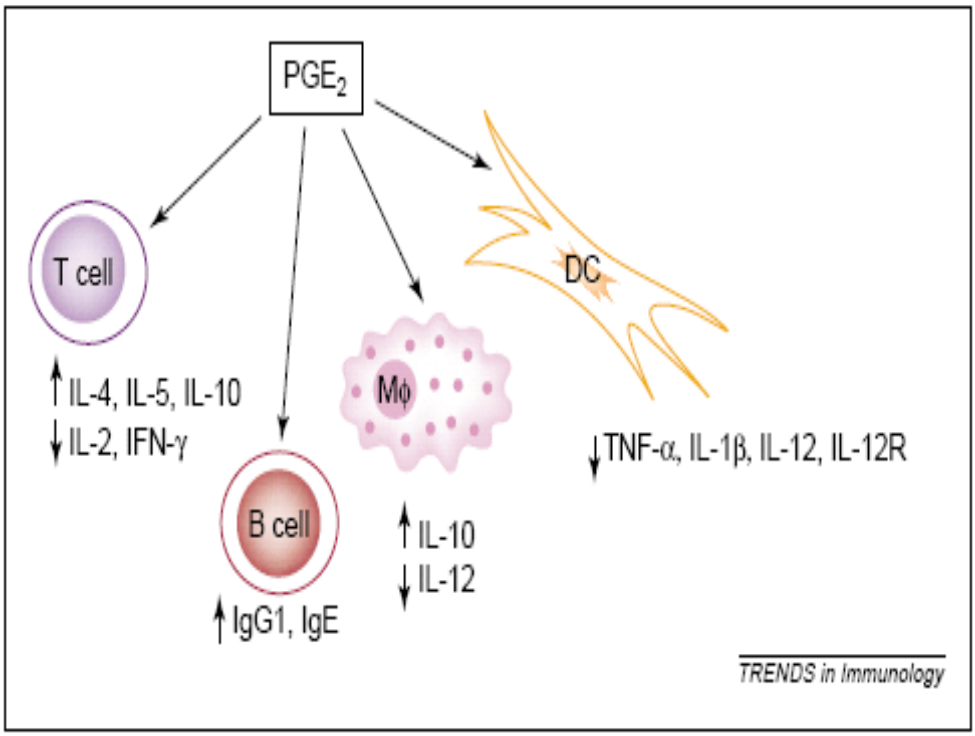

Figure 15. PGE_2 regulates the Th1/Th2 balance

PGE2 acts on T cells, B cells, macrophages, and DCs to enhance the Th2 response while inhibiting Th1 responses [152].

decidualization [157]. Chabot et al. reported major increases of endometrial PGE_2 concentrations in uterine fluid during the attachment period of porcine embryos [120]. Also, it has been shown that the placenta produces COX-2-derived PGE_2 [156], which is thought to contribute to ensuring that the maternal immune system overlooks the paternal alloantigens, thus preventing fetal rejection [156].

PART TWO

RESEARCH

1 Previous Studies, Specific Aims, Hypothesis and Approaches

1.1 Previous Studies

Previously in the lab, it has been established that the tetraspanin CD9 is the receptor for both murine PSGs 17 and 19[2] and that binding occurs between the N1 domain of these proteins [6] and the large extracellular loop of CD9 [78]. While this specific receptor-ligand interaction is required for PSG17 induced secretion of IL-10, IL-6, and TGF-β_1 in macrophages, it is not the case for human PSGs. In addition, PSG17 signals the production of arginase, and cyclooxygenase 2; COX-2 subsequently results in the secretion of PGE_2. Furthermore, inhibition of COX-2 significantly reduced the PSG17-mediated increase in IL-10 and IL-6, suggesting that COX-2 expression may be required for PSG-mediated induction of these cytokines [15].

The present research seeks to enhance our understanding of pregnancy specific glycoproteins. These studies approach the question – Are all PSGs created equal? This investigation explores the biological roles of another abundantly secreted mouse PSG, PSG23, in relation to the previously characterized PSG17.

1.2 Specific Aims

- To analyze PSG23 mediated anti-inflammatory cytokine production in macrophages. Do PSG23 and PSG17 have similar or divergent roles in pregnancy?
- To determine whether or not the biological activity of PSG23 is mediated through binding to CD9.

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[2] Ha, C. et al, unpublished data.

- Examine binding of PSG23 to other members of the tetraspanin family.

1.3 Hypothesis and Approaches

We propose that murine PSG23 binds to CD9 on macrophages and induces an anti-inflammatory cytokine response. The succeeding approaches were employed to test this hypothesis.

Prior to performing the functional and binding assays, I generated a recombinant PSG23 protein secreted from dihydrofolate reductase (DHFR) negative Chinese hamster ovary cells stably transfected with PSG23 cDNA containing both the N1 and A domains. Also, for detection and purification purposes a 6x histidine and flag tag were added to the C-terminus of the recombinant protein.

Next, PSG23 mediated cytokine production was analyzed in the murine macrophage cell line RAW 264.7 cells or peritoneal macrophages isolated from a CD9-wild type or CD9-deficient C57B/6 mouse. The cytokines were measured from the secreted supernatant or cell lysates in response to PSG23 treatment, by ELISA or real time PCR, respectively. Additionally, the effect of PSG23 on arginase and COX-2 production in RAW 264.7 cells and C57B/6 macrophages was evaluated by western blot analysis.

Various experimental approaches, including ELISA, panning, and pull-down, were utilized to determine whether PSG23 binds to CD9. Furthermore, PSG23 binding to other tetraspanins was examined by ELISA and FACS.

2 Materials and Methods

2.1 Generation of Recombinant Protein

To generate the recombinant PSG23N_1A-His-Flag protein, the cDNA consisting of the leader peptide, the N1-domain and the A-domain followed by the nucleotides coding for six histidines and the Flag tag (Sigma, St. Louis, MO) were synthesized by GenScript Corporation (Piscataway, NJ). The cDNA was excised from pUC57 with *Hind*III and *Nru*I and subcloned into the *Hind*III-*Eco*RV sites of the pEAK10 CV vector (Edge BioSystems, MD). Lipofectamine 2000 (Invitrogen Carlsbad, CA) was used to co-transfect dihydrofolate reductase negative-Chinese hamster ovary cells with PSG23N_1A-His-Flag in pEAK10 CV and the DHFR containing plasmid, pDHIP (obtained from Dr. G. Kaplan, FDA). The DHFR system allows for amplification of the Psg23 gene by gradually increasing the concentration of methotrexate (EMD Biosciences Calbiochem, San Diego, CA), leading to increased recombinant protein expression [162].

Following methotrexate selection, this protein was produced using a hollow fiber cell culture system (FiberCell Systems, Frederick, MD). The supernatant was harvested daily from the cartridge and the protein was subsequently purified by its His-tag using a His-trap affinity chromatography column (AKTA prime; GE Healthcare Life Sciences, Piscataway, NJ) followed by purification with α-Flag M2 agarose beads (Sigma).

2.2 Animals and Cell Culture

Five to seven week old C57BL/6 mice were purchased from the NCI laboratories (Frederick, MD). The CD9 deficient mice were bred from a CD9 +/- pair [15]. The mice were placed in cages with filter tops and were given *ad libitum* access to standard chow and water. Thioglycollate elicited peritoneal macrophages were obtained and cultured as previously described [17].

All cell cultures were maintained at 37°C and 5% CO_2. RAW 264.7 cells (American Type Culture Collection, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium (DMEM) with high glucose and pyruvate (Invitrogen), 100 U/ml penicillin, 100 μg/ml streptomycin (1X PS; Invitrogen), and 10% fetal clone III (VWR, West Chester PA). Baby hamster kidney (BHK)-21 cells (American Type Culture Collection) were maintained in DMEM with 10% fetal bovine serum (FBS; Hyclone), 1X PS, and 10 mM hepes (VWR). Human embryonic kidney (HEK) 293T cells (Edge Biosystems) were sustained in DMEM, 10% FBS, and 1X PS. Chinese hamster ovary (CHO) cells stably expressing PSG23N_1A-His-Flag were maintained in Iscove's modification of DMEM (Cellgro Mediatech, Inc., Herndon, VA), 2% dialyzed FBS, 0.5X PS, and 1.28 μM methotrexate (EMD Biosciences Calbiochem). After methotrexate selection the cells were cultured in DMEM, 2% FBS, 0.5X PS, and 10 mM hepes. The cells were grown in a 5 kDa MW cutoff cartridge (FiberCell Systems) and adapted to grow in 2% FBS by sequentially reducing the amount of serum. Once the cells depleted glucose in the media to half its initial concentration, the supernatant was harvested daily.

2.4 Cytokine ELISA

RAW 264.7 cells and peritoneal macrophages were seeded in a 24-well tissue culture treated plate at a density of 1 x 10^6 or 1.5 x 10^6 cells per well, respectively. After 24 hours, the cells were treated with PSG23 in triplicate at 37°C in 300 μl of media. After 3 hours, media was added to each well for a final volume of 1 ml per well. Supernatants were harvested 24 hours post treatment. ELISA was used to measure the levels of secreted TGF-β_1 (R&D Systems), and TNF-α (BD Biosciences).

2.5 Real Time PCR

RAW 264.7 cells were seeded in a 24-well tissue culture treated plate at a density of 1 x 10^6 cells per well and treated with PSG23 or PBS. Total RNA was harvested at 1.5, 2, or 4 hours post treatment using RNeasy mini kit (Qiagen, Valencia, CA), followed by cDNA synthesis with a high capacity cDNA RT kit (Applied Biosystems). Mastermix and primer assays (HPRT, IL-6, IL-10, IL-12, and IL-23; Applied Biosystems) were utilized and all the quantitative real time (QRT)-PCR reactions were completed using the 7500 Real-Time PCR system (Applied Biosystems). The QRT-PCR data was analyzed by sequence detection software, version 1.2.3 for the 7500 system SDS software (Applied Biosystems).

2.6 Western Blot Analysis

RAW 264.7 cells and peritoneal macrophages were seeded in a 24-well tissue culture treated plate at a density of 1 x 10^6 or 1.5 x 10^6 cells per well, respectively. The cells were treated for 24 hours with PSG23 and then the cell lysates were obtained by adding 100 µl of RIPA buffer with protease inhibitors. Total protein concentration was determined using BCA Protein assay kit (Pierce, Rockford, IL). Next, 25 µg of protein was loaded per lane of a 4-12% NuPage gel (Invitrogen), transferred to a PVDF membrane, blocked with 5% milk, and incubated at 4°C overnight with anti-Arginase I (BD Biosciences) or anti-COX 2 (Upstate Biotechnology, Lake Placid, NY). The amount of protein loaded per well was normalized using an anti-GAPDH monoclonal antibody (Research Diagnostics, Concord, MA). The antibodies were detected after incubation with the specific HRP-conjugated secondary antibodies followed by the SuperSignal chemiluminescent system (Pierce).

2.7 Binding Assays

2.7.1 ELISA

HEK 293T cells were seeded on a poly-D-lysine coated 96-well plate at a density of 5 x 10^4 cells per well. After 3-6 hours, cells were transfected with plasmid DNA encoding CD9, CD81, CD82, CD37, CD53, CD151, or GFP using Fugene 6 (Roche, Indianapolis, IN) as recommended by the manufacturer. Transfection efficiency was monitored by fluorescent microscopy using a green fluorescent protein (GFP) encoding plasmid. Binding was determined by ELISA 48 hours post transfection as previously described [6] using 0.73 μg/ml of anti-Flag horseradish peroxidase (HRP) conjugated antibody (Sigma).

2.7.2 Panning

Petri dishes (60 mm) were coated with 10 μg/ml of goat anti-mouse (KPL, Gaithersburg, MD) in phosphate coating solution (KPL) and incubated at room temperature on a rocker. After 4 hours, the plates were washed 4 times with 5ml of PBS and blocked with BSA buffer (KPL) overnight at 4°C. The plates were subsequently coated with 1 μg/ml of mouse anti-Flag (GenScript Corp., Piscataway, NJ) in BSA buffer for 4 hours at room temperature. The plates were washed with PBS and coated with 10 μg of either PSG23-His-Flag or PSG17-His-Flag diluted in BSA buffer overnight at 4°C. HEK 293T cells transfected with CD9 or an empty GFP encoding plasmid were added to protein coated dishes for 1 hour at room temperature. After washing the plates 6-10 times with 2% BSA in PBS, the number of adherent cells were visualized under a microscope.

2.7.3 Pull-down

The pull-down assay was performed using the ProFound pull-down poly-His protein interaction kit (Pierce) according to the recommendations of the manufacturer.

Either 150 μg of the His-tagged PSG23 or controls were added to the immobilized cobalt chelate resin. After washing, 190 μg of murine GST-CD9-EC2 was added to the immobilized bait protein. The bait-prey protein interactions were eluted from the cobalt chelate resin using 250 mM imidazole and 20 μl of the eluted material was loaded on a 4-12% NuPage gel (Invitrogen), transferred to a PVDF membrane, blocked with 5% milk, and incubated at 4°C overnight with both an anti-CD9 KMC8 antibody (Fitzgerald, Concord, MA) and an anti-GST antibody (Santa Cruz Biotech, CA). The antibodies were detected after incubation with HRP-conjugated secondary antibodies followed by the SuperSignal chemiluminescent system (Pierce).

2.7.4 FACS analysis

HEK 293T cells or BHK-21 cells were transfected with murine CD151 or transfection reagent alone using Fugene 6 (Roche) or Lipofectamine Plus (Invitrogen), respectively. Forty-eight hours (for BHK-21) or 72 hours (for HEK 293T) post transfection, the cells were treated with 10 μg of PSG23 for 1 hour on ice. The 52 KDa multiple tag protein, which includes the Flag epitope, (Genscript) was used as a control for the experiment with BHK-21 cells. After washing 3 times with wash buffer (PBS, 3%FBS, 0.01% sodium azide) the cells were consecutively incubated for 1 hour on ice with 1 μg of mouse anti-Flag mAb (Genscript), 1 μg of biotin-labeled goat anti-mouse IgG2 Ab (Molecular Probes, Eugene, OR), and 0.6 μg of Strepavidin-PE (BD Biosciences). The cells were washed 3 times with wash buffer between the incubations and were subsequently fixed using cytofix (BD Biosciences). PSG23 binding to HEK 293T and BHK 21 cells was analyzed using an EPICS XL-MCL flow cytometer

(Beckman Coulter) and the overlays were produced with the WinList program (Verity Software House).

2.8 Statistical Analysis

Data were representative of at least three independent experiments with similar results. SPSS software was used to analyze statistical significance by one-way analysis of variance (ANOVA; Chicago, IL). Data were expressed as the mean ± standard error and the mean difference is significant with $P < 0.05$.

3 Results

3.1 PSG23 induces the secretion of TGF-β_1 and IL-10 in murine macrophages in a CD9-independent manner

Prior studies demonstrated that human PSG1, 6, and 11 [16] and murine PSG17 induce IL-10, IL-6, and TGF-β_1 in human monocytes and murine macrophages, respectively. Additionally, PSG17 induces the production of arginase and cyclooxygenase 2 [15]. This research attempts to determine whether PSG23 has similar biological effects to those previously described for other PSGs.

Real time PCR was employed to analyze the effects of PSG23 on IL-10, IL-6, IL-12, and IL-23 gene expression in RAW 264.7 cells in a dose dependant and time dependant manner. RAW cells were treated with varying concentrations of PSG23 or PBS for 1.5, 2, and 4 hours and then cytokine expression was analyzed by real time PCR. PSG23 failed to upregulate IL-6 expression (*data not shown*); however, it did successfully induce IL-10 expression. The aforementioned induction is both dose and time related; as IL-10 fold induction is higher as the concentration of PSG23 used to treat the cells is increased, with peak expression between 90 minutes and 2 hours (Figure 16).

Furthermore, PSG23 treatment did not cause an increase in the expression of the pro-inflammatory cytokines, IL-12 and IL-23, compared to PBS treated cells.

PSG23 dependant COX-2 and arginase production was analyzed in RAW cells and in C57B/6 peritoneal macrophages. Cells were treated for 24 hours and cell lysates were analyzed by western blot using specific antibodies. PSG23 did not up-regulate COX-2 expression at 5, 10, or 20 μg/ml nor it increased the expression of arginase I at 25 or 50 μg/ml (Figure 17). These results suggest that the observed PSG23-mediated induction of IL-10 is not COX-2 dependant and is probably the result of another signaling mechanism.

PSG23 mediated induction of TGF-β_1 and TNF-α was analyzed in RAW 264.7 cells or C57B/6 peritoneal macrophages by ELISA. Cells were treated in triplicate with PSG23 at 5, 25, or 50 μg/ml for 24 hours at which point, the supernatants were harvested. The cytokines in the supernatant were measured by commercially available specific ELISA. Here we show that PSG23 induces the secretion TGF-β_1 but does not induce TNF-α, keeping in line with previous results seen in the lab. Moreover, PSG23 induced TGF-β_1 secretion was apparent in peritoneal macrophages prepared from CD9 deficient mice, strongly suggesting that PSG23 does not use CD9 as its receptor or that it has a second receptor on these cells (Figure 18).

3.2 CD9 is not the receptor for PSG23. PSG23 shows preferential binding to the tetraspanin CD151.

Various assays developed in our laboratory, such as pull-down, panning, and ELISA, were employed to determine whether PSG23 binds to CD9. For the poly-His pull-down assay, 150 μg of either the His-tagged PSG23 or controls were added to the

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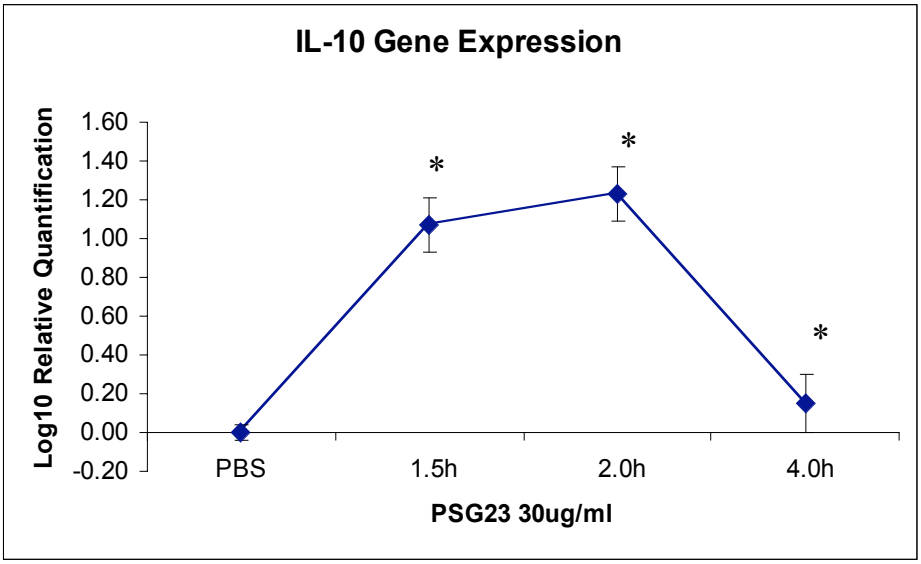

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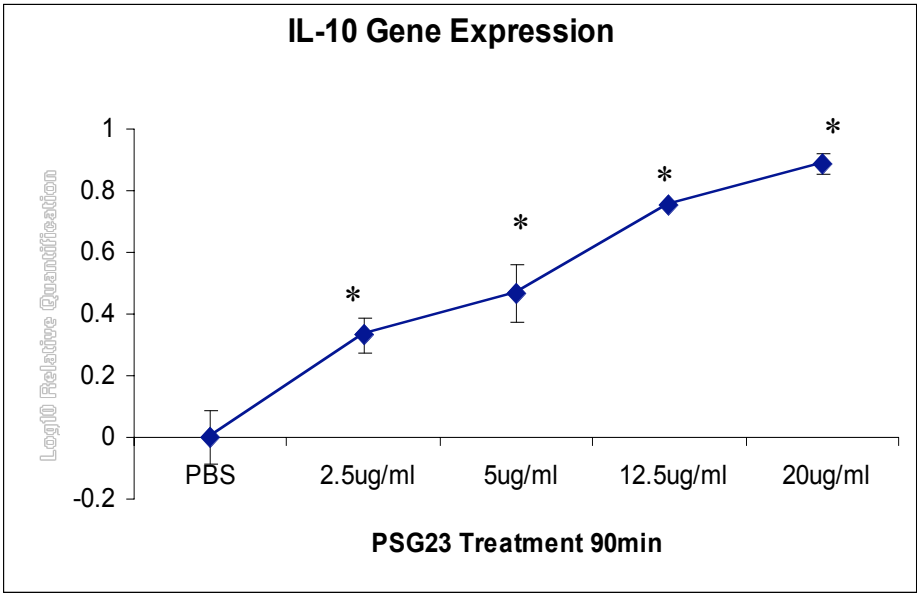

Figure 16. PSG23 induces IL-10 in murine macrophages

RAW 264.7 cells treated with PSG 23 induced IL-10 expression in a time (A) and dose (B) dependant manner. IL-10 levels are directly proportional to increased PSG levels, with peak expression between 90 minutes and 2 hours. . (*, $P < 0.05$, compared to PBS treated cells)

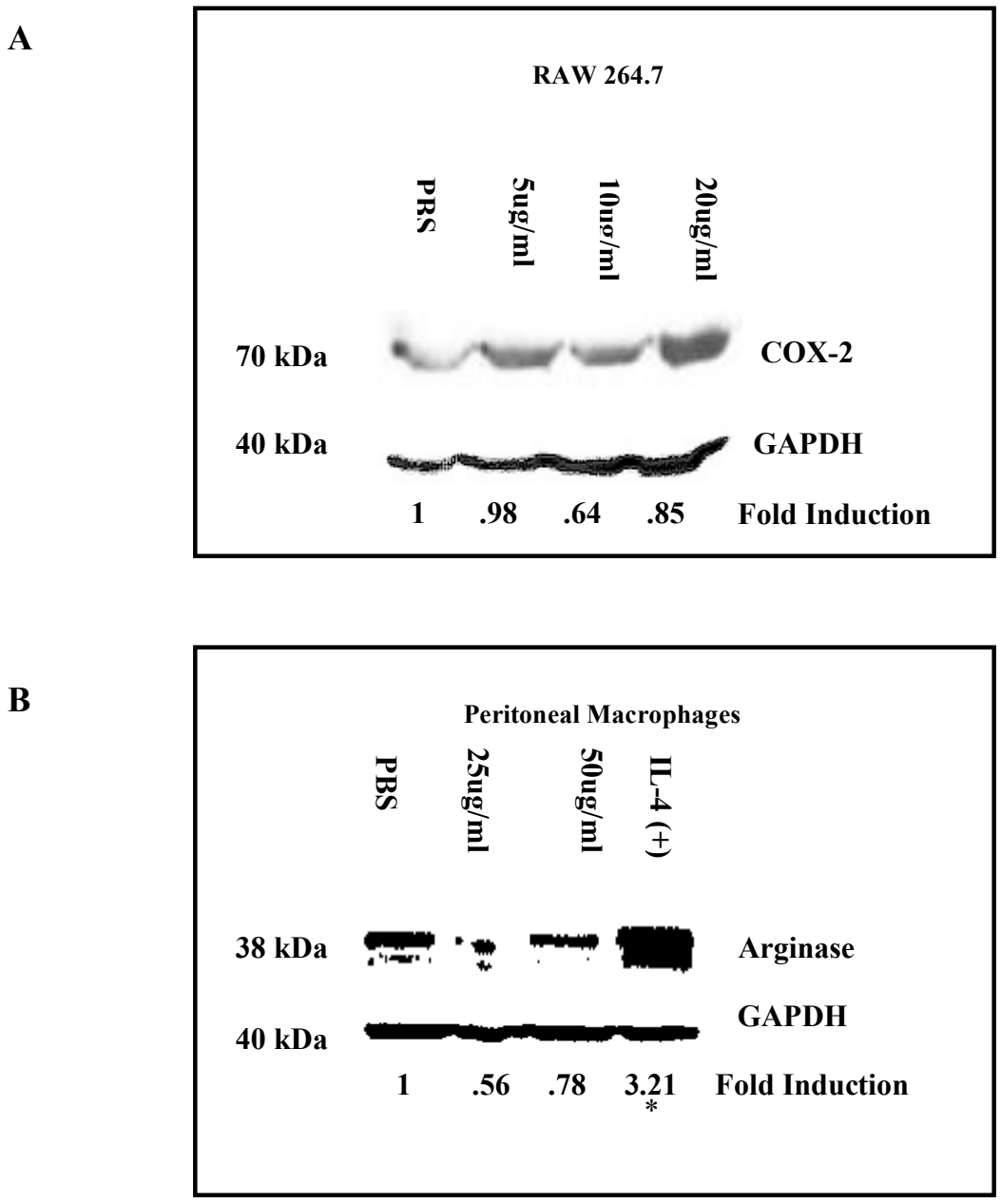

Figure 17. PSG23 does not induce production of COX2 and Arginase

RAW 264.7 cells or C57B/6 peritoneal macrophages treated with PSG23 did not up-regulate COX-2 expression at 5, 10, or 20 μg/ml (A), or arginase at 25 or 50 μg/ml (B), respectively. IL-4 was used as a positive control for arginase induction (*, $P < 0.05$, compared to PBS treated cells)

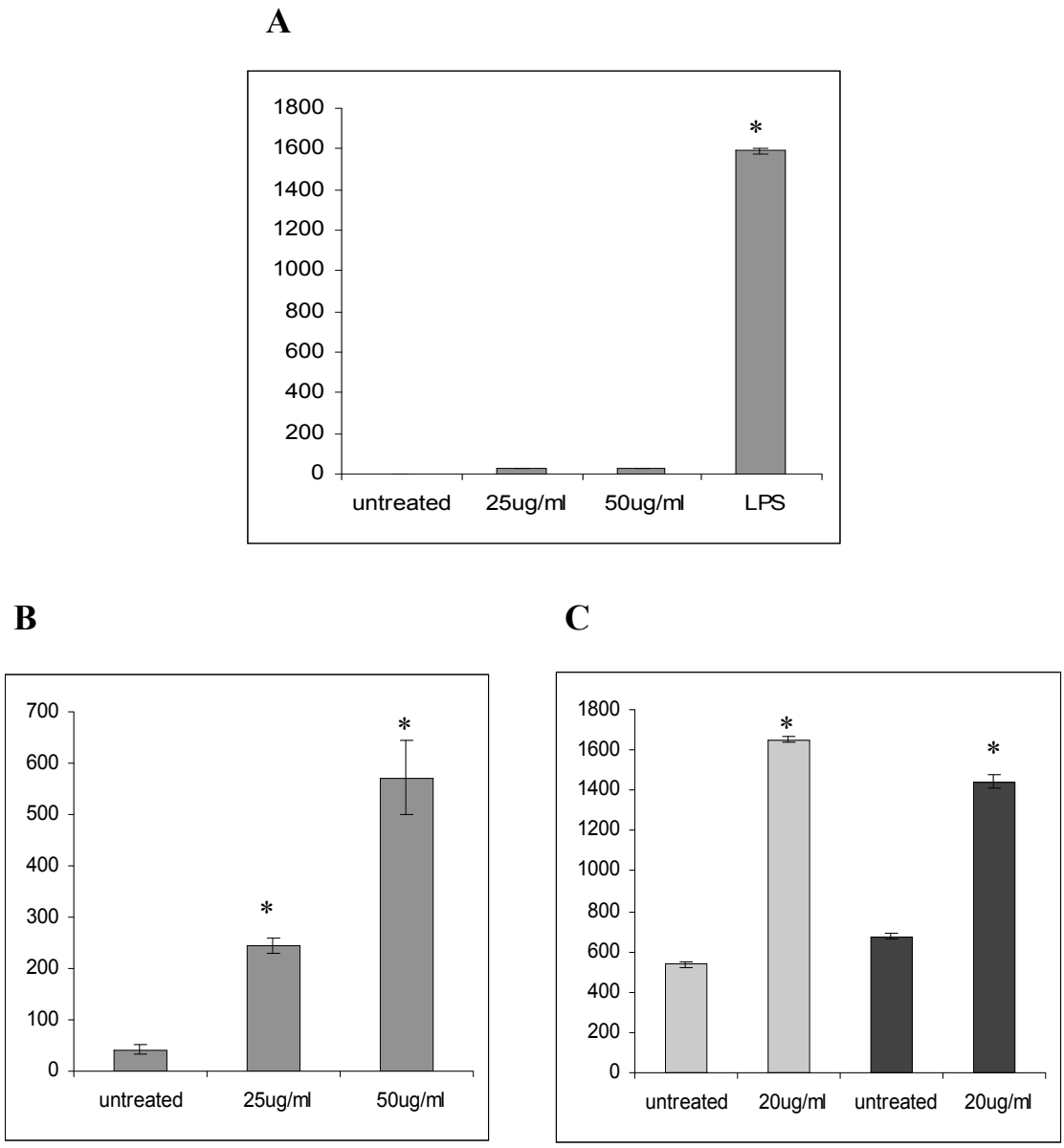

Figure 18. PSG23 induces TGF-β_1 independent of CD9 expression

(A) Treatment of RAW 264.7 cells with PSG23 does not induce the secretion of the pro-inflammatory cytokine, TNF-α; (B) however, it significantly induces TGF-β_1 secretion. (C) PSG23 induced TGF-β_1 secretion in CD9 WT (gray bars) and CD9 KO peritoneal macrophages (black bars), suggesting that PSG23 doesn't use CD9 as its receptor. . (*, $P < 0.05$, compared to untreated cells)

immobilized cobalt chelate resin, followed by 190 μg of murine GST-CD9-EC2. The bait-prey protein interactions were then eluted from the resin with 250 mM imidazole and analyzed by Western blot using both an α-CD9 and α-GST antibody. The results shown in Figure 19 indicate that CD9 was not detected in the eluted material, suggesting that PSG23 doesn't bind to CD9 (Figure 19).

For the panning experiments, 60 mm petri dishes were coated with a goat-α-mouse antibody from KPL, followed by mouse-α-Flag mAb, and 10 ug of PSG23 or PSG17 overnight at 4°C. HEK 293T cells transfected with either murine CD9-GFP or an empty GFP control plasmid were added to the protein-coated dishes for 1 hour at room temperature. The CD9 treated plates were analyzed in comparison to the controls by fluorescent microscopy. The same number of CD9 transfected cells and control cells, 3 to be exact, bound to the PSG23 coated dishes, probably due to non-specific stickiness. However, as expected, significantly more CD9 transfected cells bound to the PSG17 coated dishes when compared to the control. Again, these results confirm the interaction of PSG17 with CD9 while they suggest that CD9 is not the receptor for PSG23.

For the ELISA binding experiments, PSG23 or control PSG17 were added in triplicate to the wells expressing CD9 or empty plasmid, followed by an α-Flag-HRP conjugated antibody, TMB substrate, and (2N) Sulfuric acid (H2SO4). The color change was quantitated at 450 nm in an ELISA reader. No difference was observed between the PSG23 treated CD9 wells and control plasmid wells (Figure 20). These results agree with those previously described (see above) indicating that PSG23 does not use CD9 as its receptor.

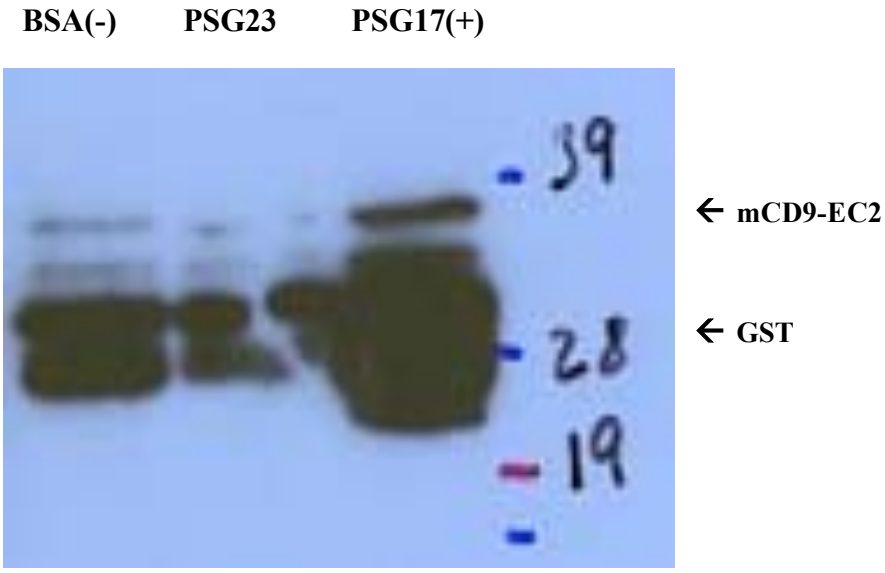

Figure 19. PSG23 does not bind to CD9 by pull-down

150 µg of PSG23 or controls were added to the immobilized cobalt chelate resin, followed by 190µg of GST-CD9-EC2. The bait-prey protein interactions were then eluted from the resin with 250 mM imidazole and analyzed by Western blot using both α-CD9 and α-GST antibodies. These results show that PSG23 does not bind to CD9 compared to the positive, PSG17, and negative, BSA, controls.

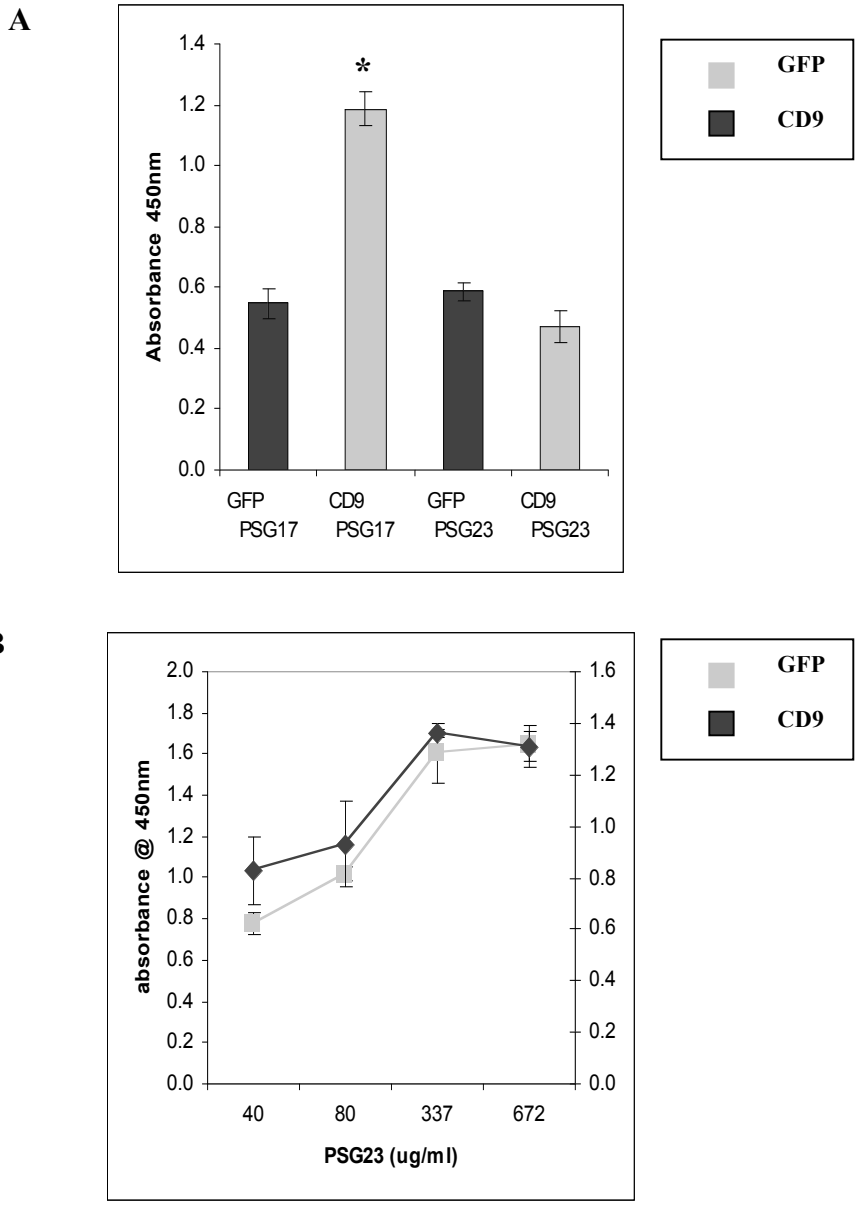

Figure 20. PSG23 does not bind to CD9 by ELISA

(A) 10 µg of PSG23 or control PSG17 were added to the cells, seeded in triplicate wells expressing CD9 or empty plasmid. Bound protein was detected using an α-Flag-HRP antibody (0.73 µg/ml). The color change was quantitated at 450 nm in an ELISA reader. There is no difference between the PSG23 treated CD9 expressing cells when compared to the control plasmid expressing cells. (B) No difference can be seen between the CD9 transfected cells and the GFP control cells with increasing concentrations of PSG23. (*, $P < 0.05$, compared to control)

We then decided to examine the possible interaction between PSG23 and other tetraspanins expressed in RAW 264.7 cells. ELISA was performed as previously described using HEK 293T cells transfected with CD81, CD82, CD37, CD53, CD151, or control GFP-encoding plasmids. Results indicate that PSG23 binds to the cells overexpressing CD151, compared to control cells (Figure 21).

FACS analysis was utilized to further confirm binding. BHK-21 or HEK 293T cells were transfected with murine CD151 or transfection reagent alone. Forty-eight hours post transfection; cells were harvested, treated with 10 μg of PSG23 or a control multi-tag protein, and detected using an α-flag antibody, followed by goat-α-mouse-biotin and strepavidin-PE. Data reveals that PSG23 does in fact bind to CD151, suggesting that PSG23 may function through receptor binding to the tetraspanin CD151 (Figure 22).

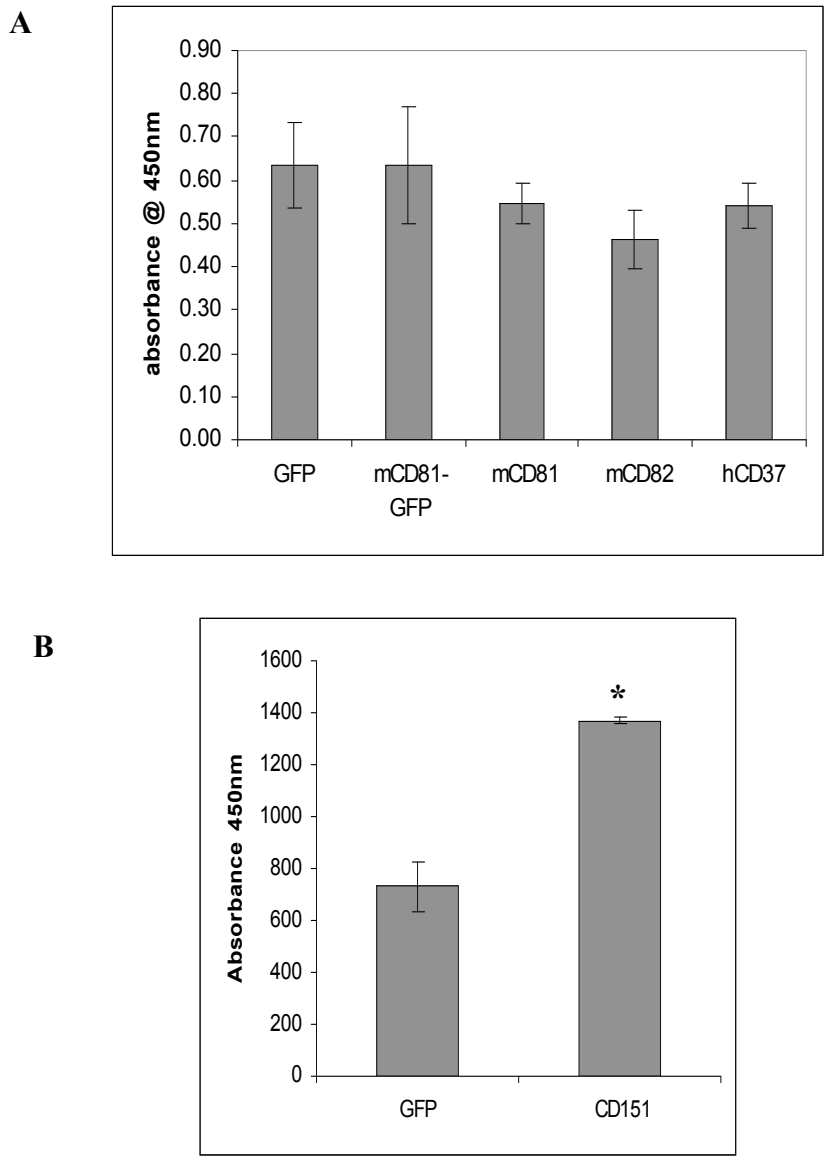

Figure 21. PSG23 binds to CD151 by ELISA

5 μg of PSG23 was added in triplicate to the wells expressing GFP control, CD81, CD82, CD37, or CD151 and bound protein was detected using an α-Flag-HRP antibody (0.73 μg/ml). The color change was quantitated at 450 nm in an ELISA reader. PSG23 bound to the cells overexpressing CD151 (shown in part B), but not CD81, CD82, or CD37, compared to control (shown in part A). (*, $P < 0.05$, compared to control)

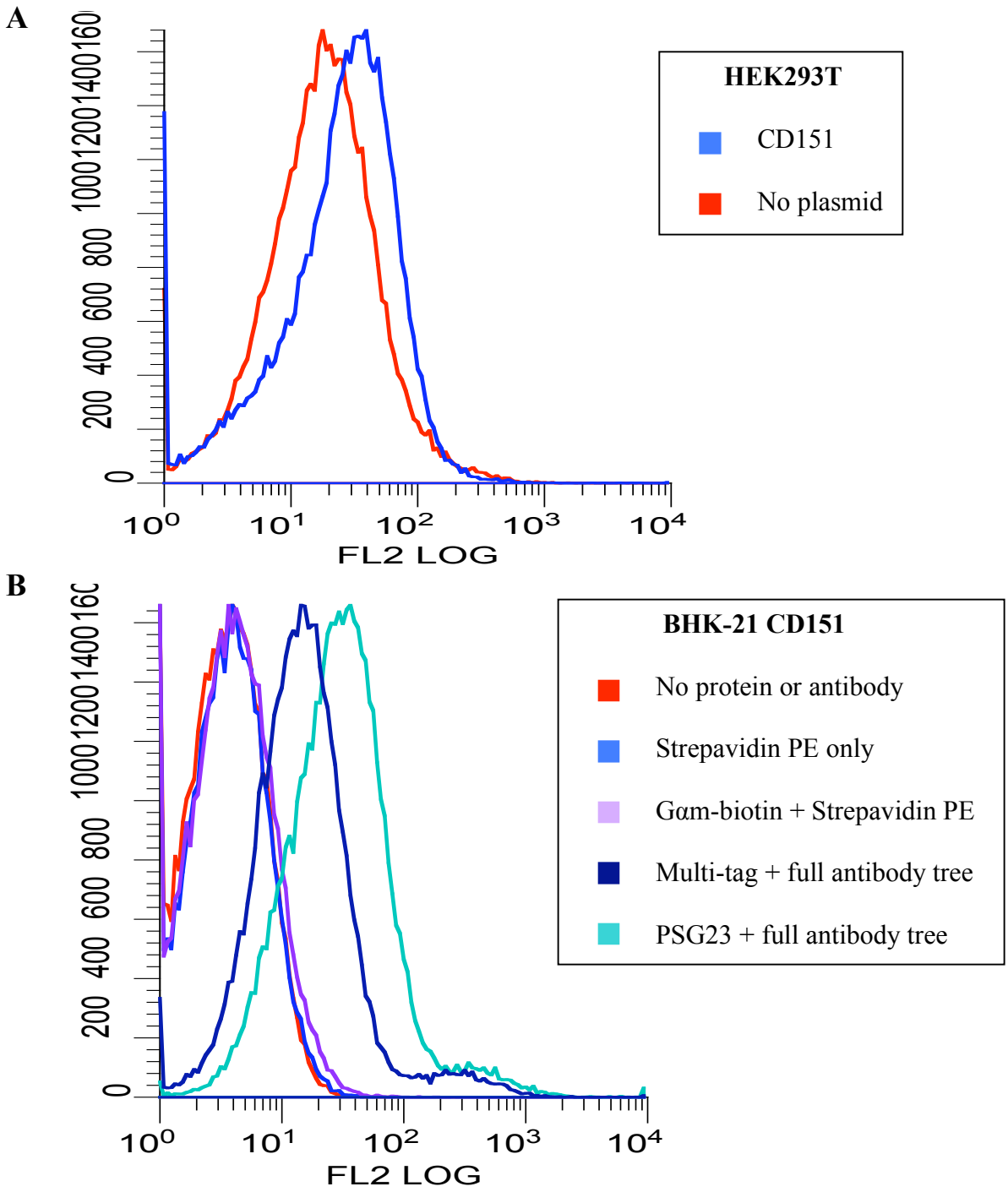

Figure 22. PSG23 binds to CD151 by FACS

HEK 293T or BHK-21 cells were transfected with murine CD151 or transfection reagent alone. Forty-eight hours post transfection; cells were harvested, treated with 10 µg of PSG23 or a control Multi-tag protein, and detected using an α-flag antibody, followed by goat-α-mouse-biotin and strepavidin-PE. Data reveals that PSG23 does in fact bind to CD151, suggesting that PSG23 may function through receptor binding to the tetraspanin CD151.

PART THREE

DISCUSSION

1 Summary of Results

This exploration attempted to enhance our understanding of the role PSGs play in maintaining a hospitable environment for fetal growth and development during pregnancy. More specifically, the biological role of murine PSG23 was examined to determine whether this specific PSG has similar roles to human and murine PSGs previously analyzed.

The results of the functional assays concur with those previously seen. Macrophages treated with recombinant PSG23 induce the anti-inflammatory cytokines TGF-β_1 and IL-10, creating a Th2 environment favorable for maternal fetal tolerance [110]. Additionally, PSG23 did not up-regulate the secretion of, TNF-α, IL-12, or IL-23, which can be detrimental to pregnancy maintenance; elevated TNF-α levels are implicated in the onset of spontaneous abortions [163]. Macrophage cytokine induction in response to PSG23 treatment is not mediated through receptor binding to the tetraspanin CD9. PSG23 successfully induced the secretion of TGF-β_1 in CD9-deficient macrophages similar to the level of induction seen in CD9 wildtype macrophages. ELISA, panning and poly-His pull-down confirmed that like the human PSGs, PSG23 does not bind to CD9. FACS and ELISA did however show preferential binding of PSG23 to the tetraspanin CD151.

2 Discussion

2.1 PSG23 induces IL-10 and TGF-β_1, creating a Th2 environment

Regulation of cytokines is important for the maintenance of pregnancy as well as labor induction. While labor is typically characterized by the infiltration of pro-inflammatory cytokines, a Th2 environment is required during the developmental stages

of pregnancy [164]. Both TGF-β and IL-10 serve as key elements in attaining the Th2 environment required for immune tolerance through gestation. As previously described, IL-10 and TGF-β_1 serve as immune regulators and there presence is vital for fetal existence [115, 164, 165].

While the exact function of PSGs remains elusive, their involvement in fetal immune protection is apparent. Like other human and murine PSGs [15, 16], PSG23 induces the secretion of IL-10 and TGFβ_1 in macrophages, in a dose-dependant manner. PSGs have been shown to create a Th2 environment, suggesting therapeutic roles during pregnancy as well as to persons suffering from Th1-dependant autoimmune disorders.

Treatment of macrophages with rec-PSG23 did not result in the upregulation of IL-6, COX-2, or arginase I; as it did in response to PSG17. What's more, Ha et al. clearly demonstrated that COX-2 induction is required for PSG17-mediated production of IL-6 and IL-10 [15]. This is undoubtedly not the case for PSG23, suggesting alternate signaling mechanisms for PSG-mediated cytokine gene regulation. These findings imply separate but comparable roles for PSGs throughout pregnancy.

2.2 PSG23-mediated cytokine induction is not dependant on CD9

In further support of alternate PSG-signaling pathways, the abovementioned production of TGF-β_1 and IL-10 is not mediated through binding to the tetraspanin CD9. PSG23 treatment of thioglycollate-elicited peritoneal macrophages from both wildtype and CD9 knockout C57B/6 mice resulted in the secretion of TGF-β_1, concurrent with the results seen in human PSGs [15], suggesting the use of an alternate receptor.

Consequently, panning, ELISA, and pull-down, were employed to ascertain whether CD9 is the receptor for PSG23. The results of the binding assays correlate with

the CD9 knock out experiments, confirming that CD9 is not the receptor for PSG23 in these cells, only PSG17 and 19 [15]. In accordance with the results of the binding experiments, sequence alignments of the N1 domain, which is responsible for receptor binding, indicate high homology between PSG17 and 19, but not PSG23 [36]. (Figure 23) Moreover, preliminary data shows reduced binding between CD9 and a rec-PSG17-mutant[3] protein, in which the RGD-like region of the N1 domain was mutated to mimic that of PSG23.

2.3 PSG23 binds to CD151: a possible role for tetraspanins in PSG-mediated maternal immune modulation

Since CD9, the only known PSG receptor thus far, is a member of the tetraspanin family there is a strong likelihood that other tetraspanins serve as receptors for PSGs as well. Therefore, we decided to investigate other tetraspanins (CD81, CD82, CD37, CD53, and CD151) expressed in macrophages in hopes to determine the receptor for PSG23. Interestingly, FACS and ELISA revealed preferential binding of PSG23 to the tetraspanin CD151.

Tetraspanins mediate a wide range of biological processes in various cell types through protein-protein interactions with other tetraspanins and proteins. CD151 has been shown to co-localize with CD9 (and CD63), suggesting that CD151 may be co-expressed with CD9 in particular tissues [166]. Furthermore, during pregnancy, CD151 expression has been observed in the placenta [76] and dNK cells. Human decidual NK cells are a unique subset of NK cells with an immunomodulatory profile. Koopman et al. propose

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[3] Dveksler, personal communication

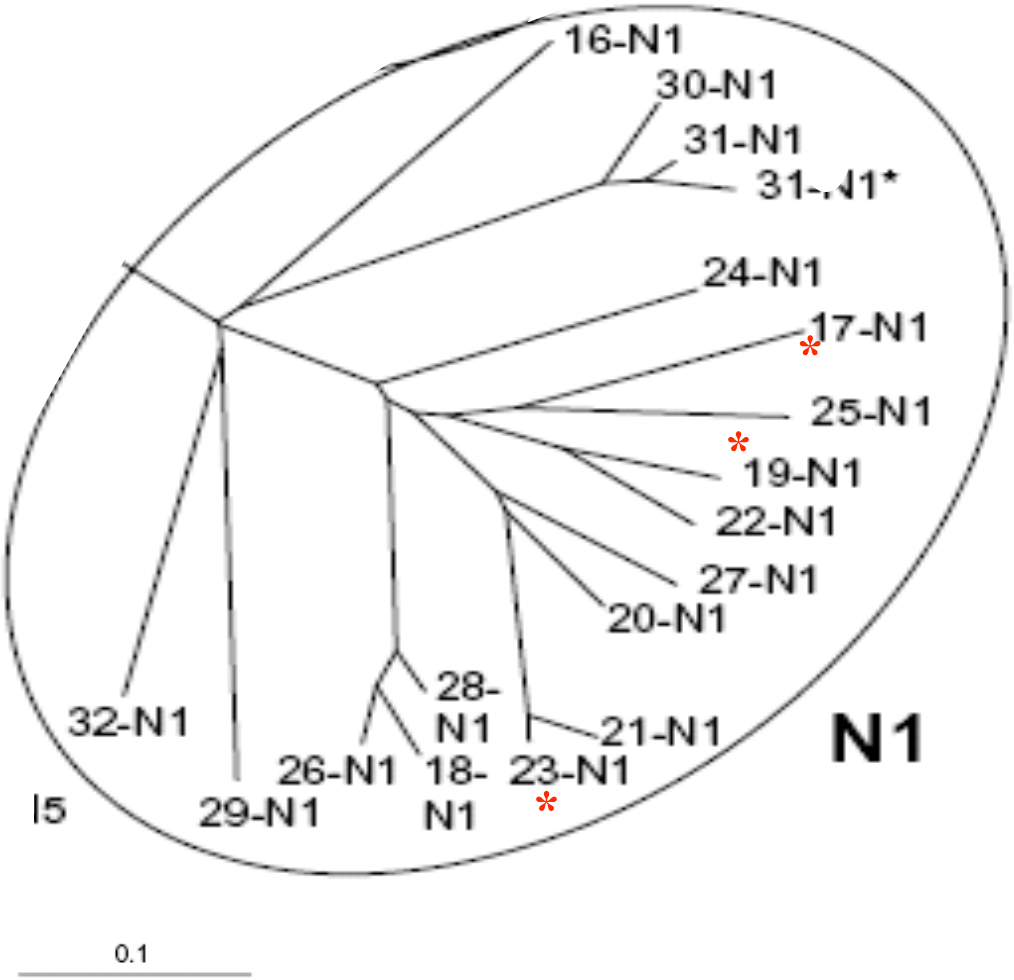

Figure 23. N1 domain of murine PSGs

Unrooted evolutionary tree based on ClustalX amino acid sequence alignments of the N1 domain. The high degree of amino acid variation with in the N1 domain of PSG23 compared to PSG17 and 19, suggests the use of different receptors. (Scale bar represents 0.1 amino acid substitutions per site) [36]

that dNK cells, through their receptor (CD9 or CD151), may interact with PSGs that subsequently produce cytokines to support successful pregnancy [73].

3 Future Directions

3.1 Examine the involvement of CD151 in PSG23 mediated cytokine induction

The present investigation illustrated a specific interaction between the tetraspanin CD151 and murine PSG23. A pull-down assay will be utilized to further confirm this proposed receptor-ligand association. Additionally, we will make use of human epithelial A431 cells, in which CD151 was silenced by siRNA, to see if there is a reduction in binding by FACS. We believe that PSG23 will not bind to these cells in the absence of CD151.

Afterward, the response of CD151-deficient cells to PSG23 treatment will be analyzed. We propose that PSG23 will not stimulate the secretion of TGF-β_1 in these cells, indicating that PSG23 functions through binding to CD151.

PART FOUR

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